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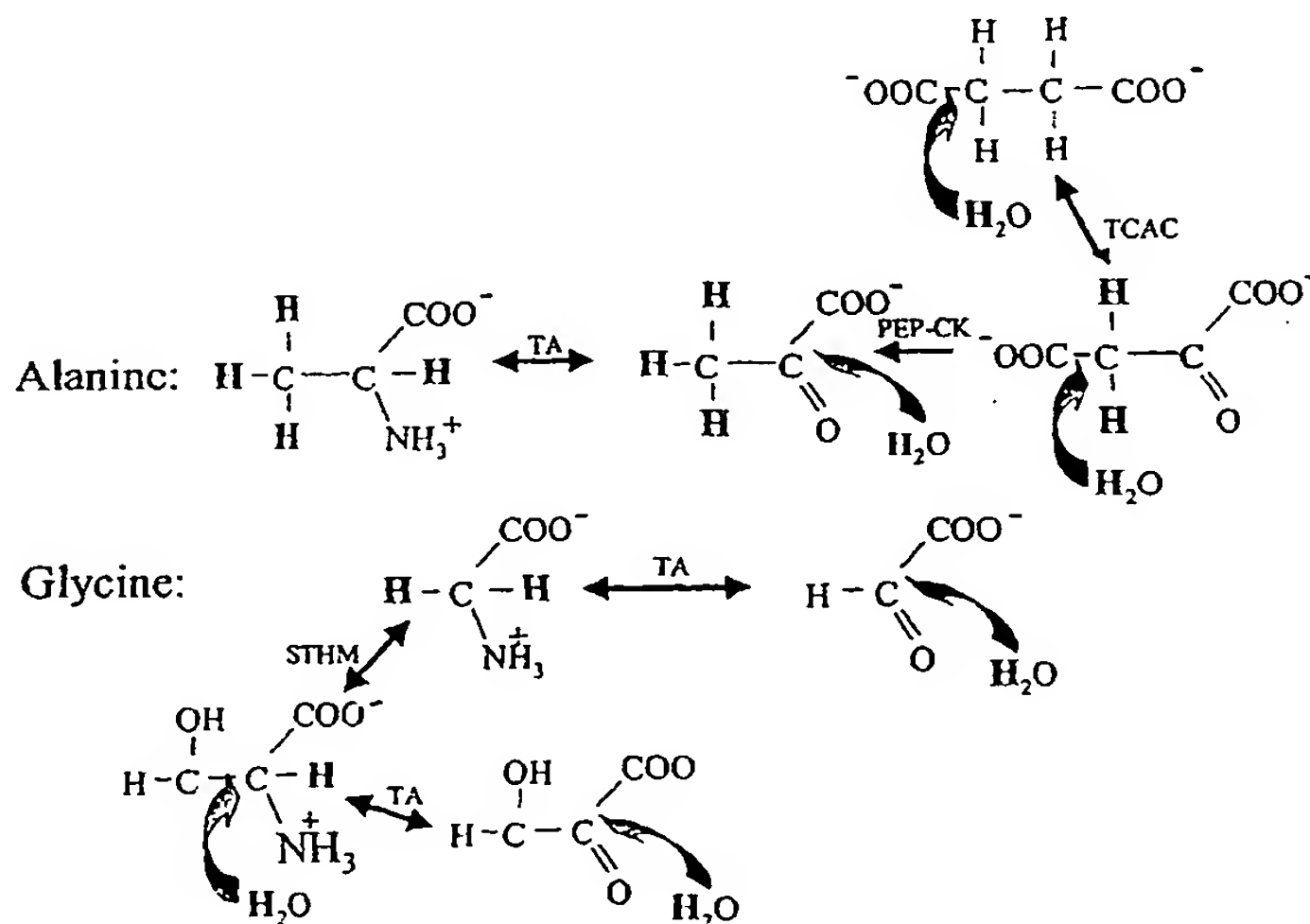
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[Continued on next page]

(54) Title: METHODS FOR COMPARING RELATIVE FLUX RATES OF TWO OR MORE BIOLOGICAL MOLECULES *IN VIVO* THROUGH A SINGLE PROTOCOL



(57) Abstract: The invention relates to techniques for measuring and comparing relative molecular flux rates of different biological molecules by administering isotope-labeled water to one or more tissues or individuals and comparing the molecular flux rates of two or more biological molecules, including biological molecules in different chemical classes. The methods find use in several applications including diagnosing, prognosing, or monitoring a disease, disorder, or condition, the *in vivo* high-throughput screening of chemical entities and biological factors for therapeutic effects in various disease models, and the *in vivo* high-throughput screening of chemical entities and biological factors for toxic effects.

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## **METHODS FOR COMPARING RELATIVE FLUX RATES OF TWO OR MORE BIOLOGICAL MOLECULES IN VIVO THROUGH A SINGLE PROTOCOL**

**[0001]** This invention was made with Government support under Grant (Contract) Nos. R01-AI43866-01AI and R01-AI43866-01AI awarded by the National Institutes of Health. The Government has certain rights to this invention.

### **Related Application**

**[0002]** This application claims priority to U.S. Provisional Patent Application Serial Number 60/484,626 filed July 3, 2003.

### **Field of Invention**

**[0003]** This invention relates to techniques for measuring and comparing relative molecular flux rates of the same or different classes of biological molecules in living systems. More particularly, it relates to techniques of administering isotope-labeled water to one or more tissues or individuals, and comparing the relative molecular flux rates of two or more biological molecules relevant to disease or to the effects of drug therapies on disease, even if the biological molecules are of different chemical classes.

### **Background**

**[0004]** In the field of drug development and discovery, there has been a long-recognized need for functional biochemical markers to validate the hypothesized phenotypic consequences of hypothesized gene or protein targets and of candidate drugs. Because of rapid technologic advances in the tools for identifying candidate targets and drugs in recent years, in particular the development of highly efficient genomic and proteomic tools for identifying potential targets of therapy (i.e., "therapeutic targets") and highly efficient combinatorial chemistry and high-throughput screening assays for generating candidate chemical or biological therapeutic leads, the need for phenotypic screening tools to eliminate ineffective or toxic candidates has become the single greatest challenge in drug development and discovery. There are too many targets and too many lead compounds for pharmaceutical companies to pursue all of them fully. Accordingly, this overwhelming number of candidate therapeutic targets and lead compounds

(see, e.g., Hellerstein and Neese, *Am J Physiol* 1999, cited in full, *infra*; Hellerstein, *Annu Rev Nutr.* 2003). Isotope labeling creates an asymmetry in time (the label at first is not present, then it is present) and thereby allows molecular kinetics to be determined.

**[0008]** Isotopic labeling techniques have typically been restricted to molecular flux rates (kinetics) of a single molecule or a single biochemical class of molecule at a time. Each labeled substrate administered is generally restricted to a single chemical class of organic molecule. By way of example, a labeled amino acid, such as  $^3\text{H}$ -leucine or  $^{13}\text{C}$ -lysine, can be given to label a protein or all proteins biosynthetically in the cell or organism of interest, but other classes of molecules (e.g., lipids, DNA, carbohydrates), are not usefully or reliably labeled from amino acids. Similarly, labels for measuring DNA and RNA kinetics do not allow kinetic measurements of lipids, proteins, and other classes of molecules. For this reason, previous kinetic labeling measurements have not provided information about relative molecular flux rates of multiple biological molecules of different classes, through a single protocol.

**[0009]** Often, it is the combinations or comparisons of different molecular flux rates that is most informative regarding biochemical consequences (phenotypes) of a drug or genetic target. (See, e.g., Hellerstein, *Annu Rev Nutr.* 2003; Stephanopoulos et al.). There is, however, a need to also analyze biomolecules from the same class but from different cell types or tissues because a comparison between the rate of proliferation of one type of cell versus the rate of another type of cell (e.g., tumor cells versus endothelial cells in various cancers) is frequently useful in evaluating therapeutic efficacy of a drug or disease diagnosis or prognosis. Accordingly, there exists a need to analyze and compare molecular flux rates of multiple classes of biological molecules concurrently in a simple, high-throughput manner. Furthermore, there exists a need to analyze biomolecules from the same class but existing in different cell types or tissues in a high-throughput manner.

### Summary of the Invention

**[0010]** In order to meet these needs, the present application is directed to a method of measuring and comparing the relative molecular flux rates of two or more biological molecules by administering isotope-labeled water. In one embodiment, the two or more biological molecules are from the same biochemical class yet are derived from different cell types or

[0013] In particular, the present application is directed to a method of measuring and comparing the relative molecular flux rates of two or more biological molecules, including when the molecules are of different chemical classes in an individual, by a) administering isotope-labeled water to an individual for a period of time sufficient for the label to be incorporated into two or more biological molecules to form two or more isotope-labeled biological molecules; b) obtaining one or more biological samples from a tissue or individual, wherein the one or more biological samples contain two or more of the isotope-labeled biological molecules; c) measuring the incorporation of the label in the two or more biological molecules to determine the molecular flux rates of the biological molecules; and d) comparing the molecular flux rates of the biological molecules to analyze their relative molecular flux rates.

[0014] Isotope-labeled water may be  $^2\text{H}_2\text{O}$ , and may be administered by any acceptable method of administration including orally, parenterally, subcutaneously, intravascularly (e.g., intravenously or intraarterially), or intraperitoneally. The individual may be a human.

[0015] Administration of isotope-labeled water may be continuous, in a single dose, or in multiple doses. The method may include the additional step of discontinuing administration of isotope-labeled water and waiting a period of time for delabeling to occur, prior to obtaining a biological sample.

[0016] In one embodiment, the biological molecules may be of different biochemical classes. In another embodiment, the biological molecules may be of the same biochemical class but derived from different cell types or tissues. In yet another embodiment, a biological molecule may be a single molecule with a defined structure.

[0017] The biological sample may be obtained pre-mortem or post-mortem. Methods of obtaining a biological sample may occur by any method, including any method of obtaining a tissue sample and any method of obtaining a biological fluid sample, including blood draw, urine collection, tissue biopsy, or other methods known in the art.

[0018] Incorporation of a label into two or more biological molecules may be detected by methods such as liquid scintillation counting, NMR, and mass spectrometry. Incorporation of isotope labels may also be detected after chemically converting biological molecules into more

third biological molecule is microglia DNA. In yet a further embodiment, a first biological molecule is amyloid precursor protein, a second biological molecule is neuron DNA, a third biological molecule is microglia DNA, and a fourth biological molecule is galactocerebroside. In another embodiment, a first biological molecule is amyloid precursor protein, a second biological molecule is neuron DNA, and a third biological molecule is microglia DNA.

[0023] Biological molecules may also be specific molecules within a class of molecules. In one embodiment, a first biological molecule is triglyceride and a second biological molecule is fatty acid, and optionally, the tissue may be liver.

[0024] The biological sample may be obtained from growing tissues such as muscle, liver, adrenal tissue, prostate tissue, colon tissue, endometrial tissue, skin, breast tissue, adipose tissue, or other tissue capable of somatic growth. The biological sample may be or include tumor cells or bacteria. The two or more biological molecules may be isolated and/or detected simultaneously.

[0025] In another aspect, the invention includes methods of detecting, prognosing, or monitoring the progression of a disease or condition in one or more tissues of individuals or in individuals. The relative molecular flux rates of two or more biological molecules in a first population of tissues or individuals that lack the disease or condition are measured and compared. The relative molecular flux rates of the two or more biological molecules in a second population of one or more tissues or individuals are measured and compared. A difference between the relative molecular flux rates between the first and the second populations is then identified and used to detect, prognose, or monitor the progression of the disease or condition. Alternatively, the relative molecular flux rates of a population of one or more tissues or individuals may be measured and compared at two or more different times.

[0026] In another aspect, the invention includes methods of detecting, prognosing, or monitoring the progression of a disease or condition in one or more tissues of individuals or in individuals. The relative molecular flux rates of two or more biological molecules in a population of tissue or individuals are measured and compared before and after administering a compound. A difference between the relative molecular flux rates before and after administration is then identified and used to detect, prognose, or monitor the progression of the



**[0030]** In a further variation, a first biological molecule is dermal collagen and the second biological molecule is dermal elastin. Photoaging may be diagnosed, prognosed, or monitored by comparing the relative molecular flux rates of dermal collagen to dermal elastin in a population of one or more individuals with a photoaging phenotype (skin wrinkles) to a test population. Alteration in the dermal collagen molecular flux rate relative to the dermal elastin molecular flux rate in the test population identifies altered photoaging in the population under evaluation. Alternatively, the relative molecular flux rates of a population of one or more tissues or individuals may be measured and compared at two or more different times.

**[0031]** The invention also includes methods of determining the efficacy of a therapeutic compound by measuring and comparing the molecular flux rates of the two or more biological molecules in a first population in need of the compound, administering the compound to the same population or to a second population of one or more tissues or individuals, and measuring and comparing the molecular flux rates of the two or more biological molecules in the same population after administration of the compound or in the second population of one or more tissues or individuals. A difference in the relative molecular flux rates of the first population before and after administration of the compound or between the first population and the second population measures the effectiveness of the therapeutic agent in tissues or individuals in need of the compound.

**[0032]** The invention also includes methods of determining the efficacy of a therapeutic compound by measuring and comparing the molecular flux rates of the two or more biological molecules in a population of one or more tissues or individuals in need of the compound, administering the compound to the same population of one or more tissues or individuals, and measuring and comparing the molecular flux rates of the two or more biological molecules in the population after administration of the compound. A difference in the relative molecular flux rates of the population before and after administration of the compound measures the effectiveness of the compound in tissues or individuals in need of the compound.

**[0033]** A tumoricidal or tumor static effect of a chemotherapeutic agent may be determined by measuring and comparing the relative molecular flux rates of cellular protein and cellular DNA. If the relative molecular flux rates of the protein and DNA do not change in

[0037] A therapeutic effect of a compound in Alzheimer's disease may be determined in one or more tissues or individuals by measuring and comparing the relative molecular flux rates of brain amyloid-beta ( $A\beta$ ) protein or amyloid precursor protein and a reference molecule in the cerebrospinal fluid (CSF). A decrease in the molecular flux rate of brain  $A\beta$  protein relative to the molecular flux rate of the CSF reference molecule (e.g., a constitutive lipid in the CSF) indicates a therapeutic effect of the compound against Alzheimer's disease. Alternatively, a therapeutic effect of a compound in Alzheimer's disease may be determined in one or more tissues or individuals by measuring and comparing the relative molecular flux rates of  $A\beta$  protein and/or amyloid precursor protein (APP), neuron DNA, and/or microglia DNA to untreated tissues or individuals or to tissues or individuals without disease (i.e., "controls"). Optionally, the molecular flux rate of galactocerebroside (either brain or plasma) may also be measured. A difference in the flux (or fluxes) of one or more of  $A\beta$ , APP, neuron DNA, microglia DNA, and galactocerebroside (or any combinations thereof) in a diseased tissue or individual (e.g., an appropriate animal model of disease) when compared to controls indicates a therapeutic effect of the compound against Alzheimer's disease.

[0038] A therapeutic effect of a compound in neuroinflammation may be determined in one or more tissues or individuals by measuring and comparing the relative molecular flux rates of neuron DNA, microglia DNA, and/or galactocerebroside (either brain or plasma) to untreated tissues or individuals or to tissues or individuals without disease (i.e., "controls"). Optionally, the molecular flux rates of one or more inflammatory proteins such as interleukin-6 and/or interleukin-12 and/or glial fibrillary acidic protein (GFAP) and/or S100B (glial calcium signaling protein) may also be measured. A difference in the flux (or fluxes) of one or more of neuron DNA, microglia DNA, galactocerebroside, interleukin-6, interleukin-12, GFAP, and S100B (or any combinations thereof) in a diseased tissue or individual (e.g., an appropriate animal model of disease) when compared to controls indicates a therapeutic effect of the compound against neuroinflammation.

[0039] A therapeutic effect of a compound in psoriasis may be determined in one or more tissues or individuals by measuring and comparing the relative molecular flux rates of keratinocyte DNA and skin keratin to untreated tissues or individuals or to tissues or individuals without disease (i.e., "controls"). A difference in the flux (or fluxes) of keratinocyte DNA

DNA relative to cellular proteins in individuals subjected to aerobic exercise identifies increased aerobic fitness.

**[0043]** The cause of a change in protein expression based on either transcriptional control or translational control may be identified by measuring and comparing the relative molecular flux rates of a protein and an mRNA encoding the protein at two or more timepoints. An increase in the molecular flux rate of the protein relative to the mRNA identifies a change in translational control, whereas a stable or decreased molecular flux rate of the protein relative to the mRNA identifies a change in transcriptional control.

**[0044]** The cause of a change in total mass or protein expression in an individual may be identified by measuring and comparing the relative molecular flux rate of total cellular RNA and total cellular DNA at two or more timepoints. An increase in the molecular flux rate of mRNA relative to the molecular flux rate of DNA identifies transcription as the cause of a change in total mass or protein expression, while no change or a decrease in the molecular flux rate of total cellular mRNA relative to the molecular flux rate of total cellular DNA identifies a change in cell division as the cause of a change in total mass or protein expression.

**[0045]** A therapeutic property of a biological agent may be identified by measuring and comparing the molecular flux rates of two or more biological molecules in a first population of one or more tissues or individuals, administering the biological agent to a second population of one or more tissues or individuals, and comparing the relative molecular flux rates of the two or more biological molecules in the two populations. A difference in the compared molecular flux rates between the two populations identifies a therapeutic property of the biological agent.

**[0046]** Alternatively, a therapeutic property of a biological agent may be identified by measuring and comparing the molecular flux rates of two or more biological molecules in a population of one or more tissues or individuals, administering the biological agent to the population, and comparing the relative molecular flux rates of the two or more biological molecules before and after the biological agent is administered. A difference in the compared molecular flux rates before and after the biological agent is administered identifies a therapeutic property of the biological agent.



**[0051]** Alternatively, toxic effects of a xenobiotic may be determined by measuring and comparing the molecular flux rates of two or more biological molecules in a population of one or more tissues or individuals, administering the xenobiotic (or combination of xenobiotics or mixtures of xenobiotics) to the population, and measuring and comparing the relative molecular flux rates of the two or more biological molecules before and after administration. A difference in the compared molecular flux rates before and after administration of the xenobiotic (or combination of xenobiotics or mixtures of xenobiotics) identifies a toxic effect of the xenobiotic (or combinations of xenobiotics or mixtures of xenobiotics).

**[0052]** In addition, the methods disclosed herein may be used to identify one or more therapeutic targets. The molecular flux rates of two or more biological molecules are measured and compared in a first population of one or more tissues or individuals. A drug or drug candidate or drug lead or new chemical entity or biological agent or already-approved drug (or any combinations or mixtures thereof) is given to a second population of one or more tissues or individuals, and the relative molecular flux rates of the two or more biological molecules in the second population are compared to the first population. A difference in the compared molecular flux rates between the first population and the second population identifies a therapeutic target.

**[0053]** Alternatively, the methods disclosed herein may be used to identify one or more therapeutic targets by measuring and comparing two or more biological molecules in a first population of one or more tissues or individuals, administering a drug or drug candidate or drug lead or new chemical entity or already-approved drug or biological agent (or combinations or mixtures thereof) to the population, and measuring and comparing the relative molecular flux rates of the two or more biological molecules before and after administration of the drug or drug candidate or drug lead. A difference in the compared molecular flux rates before and after administration identifies a drug target.

**[0054]** All methods disclosed herein are easily adaptable to high-throughput methods. A plurality of diseases and disorders, therapeutic compounds (including drugs, drug candidates, drug leads, new chemical entities, already-approved drugs or biological agents including any combinations or mixtures thereof), xenobiotics, and therapeutic targets may be screened.

[0061] **FIGURE 3** depicts enrichments of  $^2\text{H}_2\text{O}$  in body water of representative human subjects who drank 50-100 mL of  $^2\text{H}_2\text{O}$  daily for 10-12 weeks. The data show that the precursor pool of body water is stable over a period of weeks for each subject.

[0062] **FIGURE 4** depicts (a) comparison of mtDNA to nuclear DNA synthesis in cardiac and hind-limb muscle of weight-stable female rats (mean  $\pm$  S.D.); f, fractional replacement and (b) Left, synthesis of mitochondrial (mt) phospholipids in hindlimb muscle of rats, and effect of exercise training (voluntary wheel running). Animals received  $^2\text{H}_2\text{O}$  for eight days. CL, cardiolipin; PC, phosphatidyl-choline; PE, phosphatidyl-ethanolamine;  $P < 0.05$  versus control rats. Right, correlation between mtDNA and CL synthesis in rats from (a).

[0063] **FIGURE 5** depicts the simultaneous measurement of cell proliferation rates in the mature adipocyte-enriched fraction isolated from four different adipose depots (perimetrial, inguinal, mesenteric, and retroperitoneal) of control ( $n = 7$ ) and ob/ob ( $n = 4$ ) mice. Animals received 4%  $^2\text{H}_2\text{O}$  in drinking water for 21 days. Controls weighed  $18.5 \pm 0.2$  g (mean  $\pm$  S.E.) at the start of  $^2\text{H}_2\text{O}$  administration and  $20.8 \pm 0.4$  g at the end. Ob/ob weighed  $26.3 \pm 0.6$  g and  $35.7 \pm 0.9$  g, respectively.

[0064] **FIGURE 6** depicts the dynamics of adipose metabolic components in ob/ob mice and controls measured simultaneously in two different adipose depots (inguinal and mesenteric) for TG and palmitate synthesis (marker of de novo lipogenesis or DNL) and four different compartments (perimetrial, inguinal, mesenteric, and retroperitoneal) for adipocyte cell proliferation. In panel a), the effects of triglyceride synthesis are shown. In panel b), de novo lipogenesis is shown. In panel c) adipocyte proliferation is shown. The effects of food-restriction (pair-feeding) and leptin administration are compared in this manner.

[0065] **FIGURE 7** depicts skin keratin turnover in normal (C57bl/6 mice) and flaky skin mice (FSM or "flaky skin mouse," a mouse model of psoriasis) as indicated by EM1 enrichments of alanine in keratin in the two different mouse species measured simultaneously and measured simultaneously with keratinocyte DNA synthesis in both mouse species as depicted in Fig. 8. As Fig. 7 shows, keratin turnover is much more rapid (steep curve on the left) in the mouse model of psoriasis than keratin turnover (shallower curve on the right) in the normal mouse.

[0071] **FIGURE 13** depicts the fractional synthesis of cardiolipin and phosphatidylcholine in the mitochondrial heart of female Sprague Dawley rats. Cardiolipin and phosphatidylcholine were measured simultaneously. Ex = exercise. Although no changes were observed in mice that exercised for four weeks versus mice that were sedentary during that same time period, phosphatidylcholine was shown to be equivalent to cardiolipin as a biomarker of mitochondrial biogenesis.

[0072] **FIGURE 14** depicts tumor endothelial cell proliferation, liver endothelial cell proliferation, tumor cell proliferation, and total liver cell proliferation. Measurements were conducted simultaneously. See Example 15, *infra* for details.

[0073] **FIGURE 15** depicts C57B/6 total liver cell proliferation in response to two doses of carbon tetrachloride (CCl<sub>4</sub>) and to vehicle control. As Fig. 15 shows, both doses of CCl<sub>4</sub> increased total liver cell proliferation relative to vehicle control. Total liver cell proliferation was measured simultaneously with liver collagen synthesis as depicted in Fig. 16, *infra*.

[0074] **FIGURE 16** depicts C57BL/6 liver collagen synthesis in response to two doses of CCl<sub>4</sub> and to vehicle control. Measurements were conducted simultaneously with total liver cell proliferation as depicted in Fig. 15, *supra*. Fig. 16 shows that only the high dose of CCl<sub>4</sub> increased liver collagen synthesis whereas the low dose of CCl<sub>4</sub>, which increased total liver cell proliferation, had no effect on liver collagen synthesis relative to vehicle control.

[0075] **FIGURE 17** depicts C57BL/6 mouse liver cell proliferation after griseofulvin administration (0.1, 0.2 and 0.5% 5 days)  $p < 0.05$  for all groups. Fig. 17 shows increased liver cell proliferation, in a dose-dependent manner, relative to controls. The lowest dose of griseofulvin had an observable effect on liver cell proliferation. Liver collagen synthesis was measured concurrently with liver cell proliferation. Unlike CCl<sub>4</sub> (Fig. 16, *supra*), griseofulvin had no effect on liver collagen synthesis at the doses tested (data not shown).

#### **DETAILED DESCRIPTION OF THE INVENTION**

[0076] Applicants have discovered a method of measuring the relative molecular flux rates of different biological molecules, frequently in a variety of biochemical classes, through a single protocol. First, isotope-labeled water, a universal precursor, is administered to a tissue or

herein are generally well understood and commonly employed using conventional methodology by those skilled in the art, such as, for example, *Mass isotopomer distribution analysis at eight years: theoretical, analytic and experimental considerations* by Hellerstein and Neese (*Am J Physiol* 276 (*Endocrinol Metab.* 39) E1146-E1162, 1999). As appropriate, procedures involving the use of commercially available kits and reagents are generally carried out in accordance with manufacturer defined protocols and/or parameters unless otherwise noted.

[0079] "Molecular flux rate" refers to the rate of synthesis or production and breakdown or removal of a biological molecule. "Molecular flux" therefore is synonymous with the flow into and out of a pool of molecules.

[0080] "Isotopomers" refer to isotopic isomers or species that have identical elemental compositions but are constitutionally and/or stereochemically isomeric because of isotopic substitution, for example  $\text{CH}_3\text{NH}_2$ ,  $\text{CH}_3\text{NHD}$  and  $\text{CH}_2\text{DNH}_2$ .

[0081] "Isotopologues" refer to isotopic homologues or molecular species that have identical elemental and chemical compositions but differ in isotopic content (*e.g.*,  $\text{CH}_3\text{NH}_2$  vs.  $\text{CH}_3\text{NHD}$  in the example above). Isotopologues are defined by their isotopic composition, therefore each isotopologue has a unique exact mass but may not have a unique structure. An isotopologue is usually comprised of a family of isotopic isomers (isotopomers) which differ by the location of the isotopes on the molecule (*e.g.*,  $\text{CH}_3\text{NHD}$  and  $\text{CH}_2\text{DNH}_2$  are the same isotopologue but are different isotopomers).

[0082] "Mass isotopomer" refers to a family of isotopic isomers that are grouped on the basis of nominal mass rather than isotopic composition. A mass isotopomer may include molecules of different isotopic compositions, unlike an isotopologue (*e.g.*,  $\text{CH}_3\text{NHD}$ ,  $^{13}\text{CH}_3\text{NH}_2$ ,  $\text{CH}_3^{15}\text{NH}_2$  are part of the same mass isotopomer but are different isotopologues). In operational terms, a mass isotopomer is a family of isotopologues that are not resolved by a mass spectrometer. For quadrupole mass spectrometers, this typically means that mass isotopomers are families of isotopologues that share a nominal mass. Thus, the isotopologues  $\text{CH}_3\text{NH}_2$  and  $\text{CH}_3\text{NHD}$  differ in nominal mass and are distinguished as being different mass isotopomers, but the isotopologues  $\text{CH}_3\text{NHD}$ ,  $\text{CH}_2\text{DNH}_2$ ,  $^{13}\text{CH}_3\text{NH}_2$ , and  $\text{CH}_3^{15}\text{NH}_2$  are all of the same nominal

individual “at risk” may or may not have detectable symptoms indicative of the disease or physiological condition, and may or may not have displayed detectable disease prior to the treatment methods (*e.g.*, therapeutic intervention) described herein. “At risk” denotes that an individual has one or more so-called risk factors. An individual having one or more of these risk factors has a higher probability of developing one or more disease(s) or physiological condition(s) than an individual without these risk factor(s). These risk factors can include, but are not limited to, history of family members developing one or more diseases, related conditions, or pathologies, history of previous disease, age, sex, race, diet, presence of precursor disease, genetic (*i.e.*, hereditary) considerations, and environmental exposure.

[0087] “Isotope-labeled water” includes water labeled with one or more specific heavy isotopes of either hydrogen or oxygen. Specific examples of isotope-labeled water include  $^2\text{H}_2\text{O}$ ,  $^3\text{H}_2\text{O}$ , and  $\text{H}_2^{18}\text{O}$ .

[0088] “Partially purifying” refers to methods of removing one or more components of a mixture of other similar compounds. For example, “partially purifying a protein or peptide” refers to removing one or more biological molecules from a mixture of one or more biological molecules.

[0089] “Isolating” refers to separating one compound from a mixture of compounds. For example, “isolating a protein or peptide” refers to separating one specific protein or peptide from all other biological molecules in a mixture of one or more biological molecules.

[0090] A “biological sample” encompasses any sample obtained from a tissue or individual. The definition encompasses blood and other liquid samples of biological origin, that are accessible from an individual through sampling by minimally invasive or non-invasive approaches (*e.g.*, urine collection, blood drawing, needle aspiration, and other procedures involving minimal risk, discomfort or effort). The definition also includes samples that have been manipulated in any way after their procurement, such as by treatment with reagents, solubilization, or enrichment for certain components, such as proteins or polynucleotides. The term “biological sample” also encompasses a clinical sample such as serum, plasma, other biological fluid, or tissue samples, and also includes cells in culture, cell supernatants and cell lysates.



products, and the like. As used herein, the term “biologics” is synonymous with “biological factor.”

[0096] “Compound” means, in the context of the present invention, any new chemical entity, chemical entity, drug lead, drug candidate, drug, drug agent, therapeutic agent, agent, known drug, known drug agent, already-approved drug, biologic, or biological factor.

[0097] By “therapeutic target” is meant a site (e.g., a molecule within one or more metabolic pathways) within or on the body that mediates, or is thought to mediate, changes in physiology that are associated with a medical disease or condition. In many cases, a therapeutic target is unknown and the methods of the present invention allow for the discovery of one or more therapeutic targets, for example by administering one or more compounds to a tissue or individual or a population of tissues or individuals and determining changes in molecular flux rates as is more fully described, *infra*.

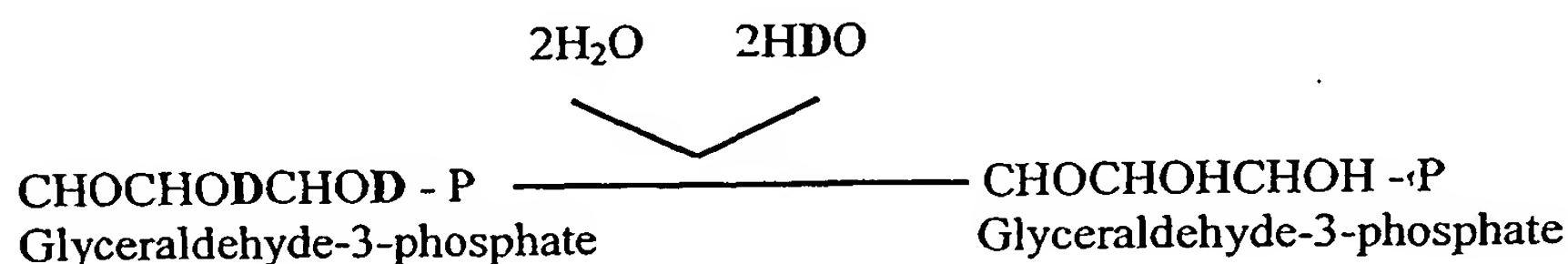
### III. Methods of the Invention

[0098] The inventor has discovered that isotope-labeled water is a universal precursor for essentially all biological molecules, especially in different chemical classes, synthesized in tissues and individuals. Introducing isotope-labeled water to a tissue or individual therefore results in the incorporation of isotope labels into biological molecules of the tissue or individual. The relative molecular flux rates of multiple biological molecules may be measured and compared. An exemplary, but not limiting list of biological molecules is provided in Table 1. Examples of biochemical processes that can be measured are provided in Table 2.

[0099] The present application is thus directed to a method of measuring and comparing the relative molecular flux rates of two or more biological molecules in an individual by a) administering isotope-labeled water to an individual for a period of time sufficient for the label to be incorporated into two or more biological molecules to form two or more isotope-labeled biological molecules, even if the two or more biological molecules are of different chemical classes; b) obtaining one or more biological samples from a tissue or individual, wherein the one or more biological samples contain two or more of the isotope-labeled biological molecules; c) measuring the incorporation of the label in the two or more biological molecules to determine the

history" – *i.e.*, label incorporation signifies that the molecule was synthesized during the period that isotope-labeled water was present in cellular water.

[00104] The labile hydrogens (non-covalently associated or present in exchangeable covalent bonds) in these biological molecules do not reveal the molecule's biosynthetic history. Labile hydrogen atoms can be easily removed by incubation with unlabelled water ( $\text{H}_2\text{O}$ ) (*i.e.*, by reversal of the same non-enzymatic exchange reactions through which  $^2\text{H}$  or  $^3\text{H}$  was incorporated in the first place), however:



[00105] As a consequence, potentially contaminating hydrogen label that does not reflect biosynthetic history, but is incorporated via non-synthetic exchange reactions, can easily be removed in practice by incubation with natural abundance  $\text{H}_2\text{O}$ .

[00106] Analytic methods are available for measuring quantitatively the incorporation of labeled hydrogen atoms into biological molecules (e.g., liquid scintillation counting for  $^3\text{H}$ ; mass spectrometry or NMR spectroscopy for  $^2\text{H}$  and  $^{18}\text{O}$ ). For further discussions on the theory of isotope-labeled water incorporation, see, for example, Jungas 1968, incorporated herein by reference.

***(2) Incorporation of isotopes from isotope-labeled water into biological molecules***

[00107] Isotope-labeled water may be administered via continuous isotope-labeled water administration, discontinuous isotope-labeled water administration, or after single or multiple administration of isotope-labeled water administration. In continuous isotope-labeled water administration, isotope-labeled water is administered to an individual for a period of time sufficient to maintain relatively constant water enrichments over time in the individual. For continuous methods, labeled water is optimally administered for a period of sufficient duration to achieve a steady state concentration (*e.g.*, 3-8 weeks in humans, 1-2 weeks in rodents).

**TABLE 1: Exemplary Biomolecules for Which Molecular Flux Rates  
Can Be Measured by the Methods of the Invention**

<u>Class</u>	<u>Examples</u>
<b>I) <u>Lipids and derivatives</u></b>	
Acylglycerides	Triglycerides Phospholipids Cardiolipin
Fatty acids	Palmitate Arachidonic acid
Sterols	Cholesterol Bile acids Estrogen, testosterone Glucocorticoids
Ceramides	Sphingomyelin Galactocerebroside
<b>II) <u>Carbohydrates and derivatives</u></b>	
Monosaccharides	Glucose Galactose
Amino sugars	N-Acetyl-Galactosamine
Polysaccharides	Glycogen
Glycoproteins	Sialic acid
Glycolipids	Galactocerebroside
Glycosaminoglycans	Hyaluronic acid Chondroitin-sulfate Heparan-sulfate
<b>III) <u>Proteins, peptides and amino acids</u></b>	
Structural proteins	Collagen Myosin
Secreted proteins	Albumin Apolipoprotein B Insulin Immunoglobulins Prostate-specific antigen Fibrinogen Interleukin-2
Secreted or excreted peptides	N-terminal collagen telopeptides Glutathione

**TABLE 2: Examples of Biochemical Processes that Can Be Measured  
(and Relevant Diseases) to Screen for Actions in Biological Systems**

	<u>Process</u>	<u>Disease</u>
I)	<b><u>DNA replication (cell division)</u></b>	
	Hepatocytes	Hepatitis; hepatic necrosis
	Lymphocytes (including antigen-specific T-cells)	AIDS; vaccination
	Spermatocytes	Male infertility
	Colonocytes	Colon cancer and colitis
	Mammary epithelial cells	Breast cancer
	Renal tubular cells	Nephrotoxins
	Prostate epithelial cells	Prostate cancer; BPH
	Tumor cells	Cancer, leukemia
	Vascular smooth muscle cells	Atherosclerosis
	Mitochondria	Metabolic fitness; mitochondrial diseases
	Pancreatic $\beta$ -cells	Type 2 diabetes
	Bone marrow progenitor cells	Bone marrow failure
	Keratinocytes	Psoriasis
	Endometrial cells	Endometrial cancer
	Endothelial cells	Angiogenesis
II)	<b><u>Fibrogenesis and bone deposition</u></b>	
	Liver collagen synthesis	Liver fibrosis; cirrhosis
	Lung collagen synthesis	Pulmonary fibrosis
	Cardiac collagen synthesis	Heart failure
	Renal collagen synthesis	Renal fibrosis
	Dermal collagen synthesis	Scleroderma
	Bone collagen synthesis	Osteoporosis; Paget's Disease
	Cartilage collagen synthesis	Osteoarthritis
III)	<b><u>Lipid synthesis and breakdown</u></b>	
	Adipose tissue triglycerides	Obesity; Lipodystrophy
	Serum cholesterol	Hyperlipidemia
	Brain myelination and demyelination	Multiple Sclerosis
	Mitochondrial phospholipids	Metabolic fitness
	Sterols	Gall bladder disease; dyslipidemia; hormonal disorders
IV)	<b><u>Tissue glycosaminoglycans</u></b>	
	Synovial fluid hyaluronic acid	Osteoarthritis; rheumatoid arthritis
	Synovial fluid chondroitin-sulfate	Osteoarthritis; rheumatoid arthritis
	Cartilage hyaluronic acid and chondroitin-sulfate	Osteoarthritis; rheumatoid arthritis
	Tumor hyaluronic acid	Metastatic potential
V)	<b><u>Protein synthesis (general)</u></b>	
	Immunoglobulins	Multiple myeloma; vaccination
	Albumin	Malnutrition
	Apolipoprotein B or E	Hyperlipidemia

### *Proteins as Biological Molecules*

[00111] In one embodiment, isotope labels from isotope-labeled water may be incorporated into proteins. The hydrogen atoms on C-H bonds are the hydrogen atoms on amino acids that are useful for measuring protein synthesis from  $^2\text{H}_2\text{O}$  since the O-H and N-H bonds of peptides and proteins are labile in aqueous solution. As such, the exchange of  $^2\text{H}$ -label from  $^2\text{H}_2\text{O}$  into O-H or N-H bonds occurs without the synthesis of proteins from free amino acids as described above. C-H bonds undergo incorporation from  $\text{H}_2\text{O}$  into free amino acids during specific enzyme-catalyzed intermediary metabolic reactions (Figure 1). The presence of  $^2\text{H}$ -label in C-H bonds of protein-bound amino acids after  $^2\text{H}_2\text{O}$  administration therefore means that the protein was assembled from amino acids that were in the free form during the period of  $^2\text{H}_2\text{O}$  exposure - i.e., that the protein is newly synthesized. Analytically, the amino acid derivative used must contain all the C-H bonds but must remove all potentially contaminating N-H and O-H bonds.

[00112] Hydrogen atoms from body water may be incorporated into free amino acids.  $^2\text{H}$  or  $^3\text{H}$  from isotope-labeled water can enter into free amino acids in the cell through the reactions of intermediary metabolism, but  $^2\text{H}$  or  $^3\text{H}$  cannot enter into amino acids that are present in peptide bonds or that are bound to transfer RNA. Free essential amino acids may incorporate a single hydrogen atom from body water into the  $\alpha$ -carbon C-H bond, through rapidly reversible transamination reactions (Figure 1). Free non-essential amino acids contain a larger number of metabolically exchangeable C-H bonds, of course, and are therefore expected to exhibit higher isotopic enrichment values per molecule from  $^2\text{H}_2\text{O}$  in newly synthesized proteins (Figure 1A-B).

[00113] One of skill in the art will recognize that labeled hydrogen atoms from body water may be incorporated into other amino acids via other biochemical pathways. For example, it is known in the art that hydrogen atoms from water may be incorporated into glutamate via synthesis of the precursor  $\alpha$ -ketoglutarate in the citric acid cycle. Glutamate, in turn, is known to be the biochemical precursor for glutamine, proline, and arginine. By way of another example, hydrogen atoms from body water may be incorporated into post-translationally modified amino acids, such as the methyl group in 3-methyl-histidine, the hydroxyl group in hydroxyproline or



**[00118]** The hydrogen atoms on C-H bonds of polynucleotides, polynucleosides, and nucleotide or nucleoside precursors may be used to measure polynucleotide synthesis from isotope-labeled water. C-H bonds undergo exchange from H<sub>2</sub>O into polynucleotide precursors. The presence of <sup>2</sup>H-label in C-H bonds of polynucleotides, nucleosides, and nucleotide or nucleoside precursors after isotope-labeled water administration therefore means that the polynucleotide was synthesized during this period. The degree of labeling present may be determined experimentally, or assumed based on the number of labeling sites in a polynucleotide or nucleoside.

**[00119]** Hydrogen atoms from body water may be incorporated into free nucleosides or polynucleotides. <sup>2</sup>H or <sup>3</sup>H from isotope-labeled water can enter these molecules through the reactions of intermediary metabolism.

**[00120]** One of skill in the art will recognize that labeled hydrogen atoms from body water may be incorporated into other polynucleotides, nucleotides, or nucleosides via various biochemical pathways. For example, glycine, aspartate, glutamine, and tetrahydrofolate are known precursors of purine rings. Carbamyl phosphate and aspartate, for example, are known precursor molecules of pyrimidine rings. Ribose and ribose phosphate, and their synthesis pathways, are known precursors of polynucleotide synthesis.

**[00121]** Oxygen atoms (H<sub>2</sub><sup>18</sup>O) may also be incorporated into polynucleotides, nucleotides, or nucleosides through enzyme-catalyzed biochemical reactions, including those listed above. Oxygen atoms from <sup>18</sup>H<sub>2</sub> may also be incorporated into nucleotides by oxidative reactions, including non-enzymatic oxidation reactions (including oxidative damage, such as formation of 8-oxo-guanine and other oxidized bases or nucleotides).

**[00122]** Hydrogen and oxygen labels from isotope-labeled water may be incorporated into a nucleic acid or a component thereof, such as those depicted in Table 1. The nucleic acids and nucleic acid components listed in Table 1 are merely exemplary; the isotope labels may be incorporated into any nucleic acid or nucleic acid component.

[00129] Hydrogen and oxygen labels from isotope-labeled water may be incorporated into any carbohydrate or carbohydrate derivative, such as those depicted in Table 1. The carbohydrate or carbohydrate derivatives listed in Table 1 are merely exemplary; the isotope labels may be incorporated into any carbohydrate or carbohydrate derivative.

[00130] The foregoing is merely exemplary. Isotope labels may be incorporated into any other known biological molecule.

**B. Obtaining one or more biological samples from one or more tissues or individuals**

[00131] One or more biological samples are obtained from the tissue or individual. The one or more biological samples may be obtained, for example, by blood draw, urine collection, biopsy, or other methods known in the art. The one or more biological samples may be one or more biological fluids. Biological samples may also be obtained from specific organs or tissues, such as muscle, liver, adrenal tissue, prostate tissue, endometrial tissue, blood, skin, and breast tissue. The biological sample may be from a specific group of cells, such as tumor cells or fibroblast cells. The one or more biological samples may be obtained pre-mortem or post-mortem. Biological molecules may be obtained, and optionally partially purified or isolated, from the biological sample using standard biochemical methods known in the art.

[00132] The one or more biological samples together include two or more biological molecules. For example, all biological molecules may be obtained from a single biological sample. Alternatively, one biological molecule may be obtained from a first biological sample, and another biological molecule may be obtained from a second biological sample. Two or more biological molecules may be obtained from each biological sample.

[00133] In a preferred embodiment, the biological molecules may also be of different chemical classes. For example, a first biological molecule may be mixed cellular proteins of a tissue or individual, while the second biological molecule may be genomic DNA of a tissue or individual. The two or more biological molecules may also be specific molecules within different chemical classes. As another example, a first biological molecule may be a specific protein with a specific amino acid sequence, and a second biological molecule may be a polynucleotide with a specific nucleic acid sequence.

incorporation of isotope labels into one or metabolic derivatives, hydrolysis products, or degradation products of biological molecules. The hydrolysis or degradation products may optionally be measured following either partial purification or isolation by any known separation method. Stable isotope-labeled substrates are incorporated into biological molecules comprising one or more metabolic pathways of interest. In this manner, the molecular flux rates can be determined by measuring, over specific time intervals, isotopic content and/or pattern or rate of change of isotopic content and/or pattern in the targeted molecules, for example by using mass spectrometry (discussed supra), allowing for the determination of the molecular flux rates within the one or more metabolic pathways of interest, by use of analytic and calculation methods known in the art.

[00139] Isotope labels in biological molecules may be detected simultaneously. For example, a mass spectrometer may be used to detect the ions of biological molecules and/or components thereof simultaneously, without requiring the physical separation, purification, or isolation of the different biological molecules.

#### *Mass Spectrometry*

[00140] Mass spectrometers convert biological molecules, and/or components thereof, into rapidly moving gaseous ions and separate them on the basis of their mass-to-charge ratios. The distributions of isotopes or isotopologues of ions, or ion fragments, may thus be used to measure the isotopic enrichment in two or more biological molecules.

[00141] Generally, mass spectrometers include an ionization means and a mass analyzer. A number of different types of mass analyzers are known in the art. These include, but are not limited to, magnetic sector analyzers, electrostatic analyzers, quadrupoles, ion traps, time of flight mass analyzers, and Fourier transform analyzers. In addition, two or more mass analyzers may be coupled (MS/MS) first to separate precursor ions, then to separate and measure gas phase fragment ions.

[00142] Mass spectrometers may also include a number of different ionization methods. These include, but are not limited to, gas phase ionization sources such as electron impact, chemical ionization, and field ionization, as well as desorption sources, such as field desorption,

algorithm are discussed in a number of different sources known to one skilled in the art. Specifically, the MIDA calculation methods are the subject of U.S. Patent No. 5,336,686, incorporated herein by reference. The method is further discussed by Hellerstein and Neese (1999), as well as Chinkes et al. (1996), and Kelleher and Masterson (1992), all of which are hereby incorporated by reference in their entirety.

[00149] In addition to the above-cited references, calculation software implementing the method is publicly available from Professor Marc Hellerstein, University of California, Berkeley.

[00150] A precursor-product relationship is then applied. For the continuous labeling method, the isotopic enrichment is compared to asymptotic (*i.e.*, maximal possible) enrichment and kinetic parameters of biological molecules (*e.g.*, biosynthesis rates) are calculated from precursor-product equations. For the discontinuous labeling method, the rate of decline in isotope enrichment is calculated and the kinetic parameters of biological molecules are calculated from exponential decay equations.

[00151] The fractional synthesis rate ( $k_s$ ) of biological molecules may be determined by application of the continuous labeling, precursor-product formula:

$$k_s = [-\ln(1-f)]/t,$$

where  $f$  = fractional synthesis = product enrichment/asymptotic precursor enrichment

and  $t$  = time of label administration of contacting in the system studied.

[00152] Similarly, breakdown rate constants ( $k_d$ ) may be calculated based on an exponential or other kinetic decay curve:

[00153]  $k_d = [-\ln f]/t$

**D. Comparing two or more molecular flux rates of two or more biological molecules**

[00154] The molecular flux rates of two or more biological molecules are compared. The comparison allows for the analysis of dynamic relationships between different biological molecules to be determined.

between a population of tissues or individuals that does not have the disease or disorder and a second population that does have the disease or disorder may be used to detect, prognose, or monitor the progression of the disease. For example, interstitial pulmonary fibrosis may be diagnosed, prognosed, or monitored by comparing the difference between the compared molecular flux rates of lung collagen and fibroblast DNA between one or more tissues or individuals that have interstitial pulmonary fibrosis and one more tissues or individuals that do not. Alternatively, the difference in compared molecular flux rates of two or more biological molecules in a single population of tissues or individuals at two or more times may be used to detect, prognose, or monitor the progression of the disease.

**[00159]** Hyperlipidemia may also be diagnosed, prognosed, or monitored by comparing the relative molecular flux rates of two or more of apolipoprotein B, triglycerides, phospholipids, or cholesterol in one or more tissues or individuals that have hyperlipidemia and one or more tissues or individuals that do not. In one variation, an increase in the molecular flux rate of apolipoprotein B relative to triglycerides, phospholipids, or cholesterol may be used to diagnose, prognose, or monitor familial combined hyperlipidemia. For example, the measurement may compare the ratio of apolipoprotein B synthesis to triglyceride synthesis. Alternatively, the difference in compared molecular flux rates in a single population of tissues or individuals at two or more times may be used to detect, prognose, or monitor the progression of the hyperlipidemia.

**[00160]** Alternatively, a state of reduced or impaired cellular immunity distinct from humoral immunity may be diagnosed, prognosed, or monitored by comparing the relative molecular flux rates of T or B cell DNA or proteins with plasma immunoglobulins (proteins). A decrease in the T or B cell DNA molecular flux rate relative to the plasma immunoglobulin molecular flux rate indicates specifically reduced cellular immune activation or function in the test population. Alternatively, the difference in compared molecular flux rates in a single population of tissues or individuals at two or more times may be used to detect, prognose, or monitor the progression of a state of reduced or impaired cellular immunity.

**[00161]** In addition, photoaging (skin wrinkles) may be diagnosed, prognosed, or monitored by comparing the relative molecular flux rates of dermal collagen and dermal elastin or dermal lipids. An alteration in the dermal collagen molecular flux rate relative to the elastin



Alternatively, if the relative rate of DNA synthesis to protein synthesis remained about the same after administration of the chemotherapeutic agent or in populations treated with the chemotherapeutic agent compared to untreated populations, the chemotherapeutic agent could be concluded to have a tumoricidal effect. Alternatively, the difference in relative molecular flux rates in a population of one or more tissues or individuals before and after administration of a chemotherapeutic agent measures the effectiveness of the chemotherapeutic agent.

[00165] The cidal or static effects of an antibiotic may analogously be determined by administering an antibiotic instead of a chemotherapeutic agent. By way of example, if the relative rate of DNA synthesis to protein synthesis was reduced after administration of said antibiotic or, in the population treated with said antibiotic compared to untreated populations, said antibiotic could be concluded to have a static effect on said infectious organism in said tissues or individuals. By "cidal" is meant the killing of the targeted infectious organism by the antibiotic. By "static" is meant the inhibition of growth or replication or proliferation or reproduction of the targeted infectious organism by the antibiotic. If the infectious organism is a bacterium, then the term "bacteriocidal" is applied to the antibiotic. Likewise, the term "bacteriostatic" applies to antibiotics that inhibit bacterial growth or replication or proliferation or reproduction.

[00166] In another embodiment, one or more beneficial therapeutic effects of an androgen in one or more tissues or individuals with a wasting disease or disorder of frailty may be determined. A difference in the molecular flux rate of muscle protein or DNA relative to the molecular flux rate of adipose tissue triglyceride between a population to which the androgen has been administered and a population to which the androgen has not been administered identifies or measures the beneficial therapeutic effect. Alternatively, the difference in relative molecular flux rates between a population of one or more tissues or individuals before and after administration of an androgen measures its effectiveness. An increase in the molecular flux rate of the muscle protein or DNA relative to the molecular flux rate of the adipose tissue triglyceride after therapy identifies a beneficial therapeutic effect of the androgen in the wasting disease or disorder of frailty. A beneficial therapeutic effect of other muscle anabolic factors, such as growth hormone, in a wasting disease or disorder of frailty may be identified in the same manner, by administering the growth hormone instead of the androgen.

turnover) in a population of one or more individuals before and after administration of a hormonal or other therapeutic agent. Alternatively, the beneficial therapeutic effect may be identified by comparing these relative molecular flux rates in a population of one or more individuals after administration of the hormonal or other therapeutic agent to Alzheimer's disease patients to whom the hormonal or other therapeutic agent has not been administered. A decrease in the molecular flux rate of amyloid beta protein relative to the molecular flux rate of the 25-hydroxy cholesterol identifies a beneficial therapeutic effect of the hormonal or other therapeutic agent against Alzheimer's disease.

**[00170]** The invention also includes methods of identifying a therapeutic property of a biological agent. The relative molecular flux rates of two or more biological molecules are determined in a population of one or more tissues or individuals to which a biological agent has been administered and a population of one or more tissues or individuals to which the biological agent has not been administered. A difference in the relative molecular flux rates identifies a therapeutic property of the biological agent. Alternatively, the therapeutic property may be identified by comparing the molecular flux rates in a population of one or more individuals to the same population before and after administering the agent. The therapeutic property may be an undiscovered property of an already-approved drug (i.e., an "old" drug), for example. The biological sample may be a tissue culture, and the individual may be an experimental animal or a human. Drug agents may be any chemical or biological compound or composition known in the art. Drug agents include, but are not limited to, any chemical compound or composition disclosed in, for example, the 12th Edition of *The Merck Index* (a U.S. publication, Whitehouse Station, N.J., USA), incorporated herein by reference in its entirety. The method may be used to screen a plurality of drug agents in a high-throughput manner.

**[00171]** Toxic effects of drug agents, including biological agents, may also be determined by the methods of the present invention.

#### **Multiple concurrent measurements used for screening functions**

**[00172]** Because of the great breadth of measurements that are possible using isotope-labeled water, screening for unexpected or unidentified actions of drugs or general protein targets is possible. Examples include the effects of statins (3-hydroxy-3-methylglutaryl coenzyme A

and-error method and consequences on multiple molecular flux rates as end-points have to be measurable.

#### **Capacity to evaluate long-term, slowly evolving toxicities or therapeutic actions**

[00175] Many biological processes that are important to potential toxic or therapeutic actions of candidate drugs or genes are very slow to evolve. These slowly evolving processes can be monitored by methods of administering isotope-labeled water, unlike other kinetic techniques. This is because of the extreme ease and simplicity of administering isotope-labeled water, such as  $^2\text{H}_2\text{O}$ , in drinking water.  $^2\text{H}_2\text{O}$  labeling studies have been carried out with minimal effort for 3-6 months in experimental animals and human subjects (see, e.g., Figure 3). Such slowly evolving processes that can be monitored include kinetics of vascular smooth muscle cells, lymphocytes, brain constituents, tissue mitochondria, epithelial stem cells, and bone and tissue collagen, and many others.

#### **Kits**

[00176] The invention provides kits for measuring and comparing molecular flux rates in vivo. The kits may include isotope-labeled water (particularly  $^2\text{H}_2\text{O}$ ,  $^3\text{H}_2\text{O}$ , and  $\text{H}_2^{18}\text{O}$  isotope-labeled water or a combination thereof), and in preferred embodiments, chemical compounds known in the art for separating, purifying, or isolating biological molecules, and/or chemicals necessary to obtain a tissue sample, automated calculation software for combinatorial analysis, and instructions for use of the kit.

[00177] Other kit components, such as tools for administration of water (e.g., measuring cup, needles, syringes, pipettes, IV tubing), may optionally be provided in the kit. Similarly, instruments for obtaining samples from the subject (e.g., specimen cups, needles, syringes, and tissue sampling devices) may also be optionally provided.

#### **EXAMPLES**

[00178] The following non-limiting examples further illustrate the invention disclosed herein:

an older, weight-stable female group (8-10 months of age). Initial average rat weights from the young rat group was approximately 210 g while the initial average body weights from the older, weight-stable group was approximately 225 g.

[00183]  $^2\text{H}_2\text{O}$  labeling protocols in rodents consisted of an initial intraperitoneal priming bolus to 2.0-2.5% body water enrichment. The priming dose of  $^2\text{H}_2\text{O}$  (100%) was given to the rats (e.g., for a 225g rat, 2% of 135ml, or 2.7ml, given in divided doses 1 hour apart) based on estimated 60% body weight as water, followed by administration of 4%  $^2\text{H}_2\text{O}$  in the drinking water. The 4% enrichment of  $^2\text{H}_2\text{O}$  in drinking water was chosen as a convenient dose that produces sufficient enrichments in biosynthetic products of interest and has no known toxicities.  $^2\text{H}_2\text{O}$  (70% and 100%) was purchased commercially from Cambridge Isotopes (Andover, MA). Drinking was ad-libitum. Rats were sacrificed by  $\text{CO}_2$  asphyxiation.

[00184] Bone marrow and cardiac (0.5g) and hindlimb muscle (0.3g) samples from individual animals were removed immediately after sacrifice. Muscle samples were homogenized and mitochondria from the homogenate were then isolated by density gradient centrifugation. Nuclear DNA (nDNA) contamination was removed enzymatically by treatment with DNase. Absence of nDNA contamination in muscle samples was confirmed by polymerase chain reaction (PCR) followed by gel electrophoresis. More than sufficient mtDNA was obtained from 0.3-0.5g of muscle tissue for measurement of mtDNA kinetics in individual animals.

[00185] Bone marrow nDNA was isolated by use of a Qiaamp column (Qiagen) using techniques well known in the art. MtDNA was also isolated from cardiac muscle, hindlimb muscle and platelets using the Qiagen Kit (Qiagen) after isolation of the mitochondrial fraction from the tissue. MtDNA and nDNA were hydrolyzed enzymatically to free deoxyribonucleosides. A LC18 SPE column (Supelco, Bellefonte, PA) was used to separate dA from the other deoxyribonucleosides. The column was washed with 100% methanol (2ml) and water (2ml). The hydrolyzed DNA sample was then added to the column and nucleosides other than dA were eluted with an  $\text{H}_2\text{O}$  wash (5ml). The dA was then eluted with 50% methanol (1ml).

[00186] The deoxyribose (dR) moiety of dA was analyzed by GC/MS, after conversion to its pentane-tetraacetate derivative. The isotopic enrichment of dR was determined by GC/MS

from 0.0%-3.5% over time. Fractional synthesis (f) of mt DNA from hindlimb and cardiac muscle also increased over time. Approximately 40-50% of mtDNA was newly synthesized after 11 weeks of labeling with  $^2\text{H}_2\text{O}$  in both tissues, with the value in hindlimb muscle appearing to plateau at about 45%.

[00191] In order to distinguish between mtDNA synthesis related to tissue accrual and mtDNA replacement independent of growth, we also studied older, weight-stable female rats. Body weights over time were relatively stable in these animals, as expected, with only a 10% increase over the 9 week labeling period. Bone marrow dA enrichment values were stable and not different from growing male rats. Tissues were sampled through week 9 of  $^2\text{H}_2\text{O}$  labeling. In cardiac muscle, the mtDNA synthesis (ca. 20% new) after 8 weeks of  $^2\text{H}_2\text{O}$  labeling was greater than somatic growth (ca. 10%). In skeletal muscle, mtDNA synthesis was similar in magnitude to somatic growth, so synthesis independent of growth was not demonstrated by this means. The rate constant of synthesis was about 0.4% per day in cardiac muscle. After correcting for somatic growth (10% over 9 weeks), the calculated fractional replacement rate of cardiac muscle mtDNA was about 0.2%/ day, consistent with a half-life of 350 days for mtDNA.

Comparison of mtDNA to nDNA synthesis in muscle tissue by concurrent measurement

[00192] The fractional synthesis of nDNA and mtDNA in cardiac and hindlimb muscle were compared in weight stable female rats. Higher synthesis rates of mtDNA compared to genomic DNA, by about 2-fold after 6-9 weeks, were observed in both cardiac and hindlimb muscle tissues (Fig. 5). Of note, skeletal muscle nDNA synthesis was of lower magnitude (ca 5% after 9 weeks) than whole body somatic growth (ca 10%) in these animals, whereas cardiac muscle nDNA synthesis (ca 10%) was of similar magnitude as somatic growth. The observation that mtDNA synthesis is greater than nDNA synthesis in these tissues is important because these results are consistent with mtDNA replication independent of cell division. If mtDNA synthesis is corrected for new myocyte proliferation (nDNA synthesis), the half-life of mtDNA in non-growing tissue can be estimated, as was done based on somatic growth (see above). Calculated half-life in cardiac muscle was ca. 350 days (replacement rate constant  $0.2\% \text{ d}^{-1}$ ) and in skeletal muscle was about 700 days (replacement rate constant  $0.1\% \text{ d}^{-1}$ ).



(Coulbourn Instruments, Allentown PA) which delivered one 45 mg pellet every 23 minutes continuously for the period of study. Food restriction began 5 days prior to the start of labeling with  $^2\text{H}_2\text{O}$ .

[00197] Ob-lep mice received murine leptin at a dose of  $2\mu\text{g}/\text{day}$  (Amgen, Thousand Oaks CA) via a 28 day Alzet mini osmotic pump (Alza Corp. Palo Alto CA) implanted subcutaneously. The pump delivered a continuous infusion of leptin ( $0.35\mu\text{g}/\mu\text{l}$ ) subcutaneously at a rate of  $0.25\mu\text{l}$  per hour. Leptin treatment also began 5 days prior to the beginning of labeling with  $^2\text{H}_2\text{O}$ .

[00198] Mice were injected with  $^2\text{H}_2\text{O}$  (deuterated water) at a dose to achieve approximately 2% enrichment in the body water pool. The normal drinking water was then replaced with water enriched to 4%  $^2\text{H}_2\text{O}$ .  $^2\text{H}_2\text{O}$  treatment had no impact on food intake or body weight. Twenty-one days following the start of  $^2\text{H}_2\text{O}$  administration, mice were fasted for four hours, anesthetized with isoflurane and exsanguinated via heart puncture. When possible, urine was collected at the same time.

#### Blood measurements

[00199] Plasma glucose was measured with a YSI (Yellow Springs, OH) auto analyzer. Plasma insulin and leptin assays were performed by assay services at Linco research (St. Charles, MO.).

#### Adipose tissue preparation and isolation of adipocytes

[00200] Fat pads were isolated and dissected according to the following procedure. The inguinal fat pad was defined as the discreet subcutaneous fat pad beginning at the base of the hind legs and extending up to the rib and back to the spine. The perimetrial fat pad was identified and dissected from the ovary and uterus. Mesenteric adipose was removed by stretching the intestine out and gently pulling the fat and lymph tissue away. Retroperitoneal fat pads were located behind the kidneys and extended down toward the top of the perimetrial pad, the upper limit of the uterus was used as a boundary. For inguinal and retroperitoneal pads, the left and right sides were pooled for analysis.

Derivitization and analysis of TG, FA and H<sub>2</sub>O

[00205] Samples of adipose TG were taken following the incubation with collagenase. Between 10- 20  $\mu$ L of lipid was removed and frozen in ca. 500 $\mu$ L heptane containing 0.01 % betahydroxytoluene. This solution was extracted with 2 ml chloroform: water (1:1). The aqueous phase was discarded and the lipid fraction was transesterified by incubation with 3N methanolic HCL (Sigma-Aldrich) at 55° C for 60 min. Fatty acid methyl esters were separated from glycerol by Folch extraction with the modification that water rather than 5% NaCl was used for the aqueous phase. The aqueous phase containing free glycerol was then lyophilized and the glycerol converted to glycerol tri-acetate by incubation with acetic anhydride-pyridine, 2:1, as described elsewhere (Hellerstein, M. K., Neese, R. A., and Schwarz, J. M. (1993) *Am J Physiol* 265, E814-820). The phase containing fatty acid-methyl esters was concentrated under nitrogen and injected directly into the GC/MS.

[00206] <sup>2</sup>H<sub>2</sub>O enrichments in body water were measured in tetrabromoethylene derivitized from plasma samples as described in detail elsewhere (Neese, R. A., Siler, S. Q., Cesar, D., Antelo, F., Lee, D., Misell, L., Patel, K., Tehrani, S., Shah, P., and Hellerstein, M. K. (2001) *Anal Biochem* 298, 189-195.).

GC/MS analyses

[00207] Model 5970 and 5971 GC/MS or 5973 instruments (Agilent, Palo Alto, CA) were used for measuring isotopic enrichments of glycerol-triacetate fattyacid -methylesters and tetrabromoethylene. Glycerol-triacetate was analyzed using a DB-225 fused silica column, monitoring m/z 159 and 160 (parent M<sub>0</sub> and M<sub>1</sub>), or m/z 159, 160 and 161 (M<sub>0</sub>, M<sub>1</sub> and M<sub>2</sub>). Methane chemical ionization (CI) was used with selected ion monitoring. Fatty acid-methyl esters composition was analyzed by flame ionization detection and for <sup>2</sup>H-enrichment by GC/MS. Tetrabromoacetylene was analyzed using a DB-225 fused silica column, monitoring m/z 265 and 266 (parent M<sub>0</sub> and M<sub>1</sub> masses). Standard curves of known <sup>2</sup>H<sub>2</sub>O enrichment were run before and after each group of samples to calculate isotope enrichment.

[00208] PTA samples were analyzed for incorporation of deuterium on a HP model 5973 MS with a 6890 GC and auto-sampler (Agilent, Palo Alto, CA). Methane CI was used with a

that a correction for the change in pool size is required to estimate lipolysis rates from label incorporation measurements:

$$\text{Net lipolysis} = \text{TG synthesized} - \text{TG accumulated.}$$

[00211] In the case of the leptin treated mice in which TG retained was a negative value (i.e., body fat was lost) TG synthesis rate is added to the quantity of TG lost to yield the net lipolysis rate.

[00212] DNL (fraction of total and fraction of newly synthesized TG)

[00213] MIDA was used to measure fractional DNL for palmitate from adipose TG, as described supra, and in U.S. Patent No. 5,338,686, herein incorporated by reference in its entirety.  $^2\text{H}_2\text{O}$  labeling was used.

[00214] The calculated fractional DNL measured by MIDA represents the fraction of stored TG that was synthesized via the DNL pathway during the labeling period. This value does not represent the proportion of DNL in newly synthesized fatty acid stored, however, to the extent that pre-existing fat is present. That is, "non-DNL" TG could represent either pre-existing TG or newly synthesized TG from non-DNL pathways. This problem can be solved by correction for the proportion of TG that is newly synthesized. The ratio of DNL-f to TG-f reveals the true fraction from DNL in new fat storage. If the fractional DNL contribution is 35% and the fractional TG synthesis is 70% the true fractional DNL in newly synthesized TG is  $0.35/0.7$  or 50%, rather than the 35% f measured directly.

[00215] Absolute palmitate DNL was calculated by multiplying fractional DNL by 0.8 times the weight of the fat pad (the estimated fraction of TG in adipose tissue) then by the percent palmitate present (measured by flame ionization detection). This value represents the absolute amount (grams) of palmitate synthesized during the labeling period.

Adipose cell proliferation (adipogenesis)

measured concurrently by administering labeled water and detecting the incorporation of the isotope label in protein, mRNA, and DNA.

**EXAMPLE 7: Rates of, triglyceride and fatty acid input into lipoproteins assembled by the liver.**

[00220] The assembly of very-low-density-lipoprotein (VLDL) particles in the liver underlies many forms of hyperlipidemia and is the target for hypolipidemic therapies. Hepatic VLDL assembly involves the synthesis of several classes of molecules (ApoB, cholesterol, cholesterol-esters, triglycerides, phospholipids), some of which themselves involve more than one biosynthetic pathway (e.g., synthesis of fatty acid and acyl-glyceride moieties in the assembly of triglyceride and phospholipids; synthesis of cholesterol and fatty acid moieties in the synthesis of cholesterol-esters). Different varieties of human hyperlipidemias appear to be due to alteration in different pathways on this list. The form called familial dyslipidemic hypertension (or hyperapobeta lipoproteinemia) is due to excessive secretion of ApoB. Other forms, such as carbohydrate-induced hyperlipidemias may reflect changes in rates of triglyceride synthesis, fatty acid synthesis, or removal of triglycerides from VLDL. The production rates of all of these components are measured concurrently by administering labeled water and detecting the incorporation of the isotope label in each of the biological molecules.

**EXAMPLE 8: De novo lipogenesis contribution to adipose fat accrual corrected for new adipose triglyceride synthesis.**

[00221] The contribution from de novo lipogenesis (DNL) or endogenous synthesis of new fatty acids (such as palmitate by the body) compared to dietary fat intake (ingestion of fatty acids) represents a key distinction in the physiology of body fat accumulation (e.g., obesity). The enzymes responsible for de novo lipogenesis, and therefore the drug treatment indicated, differ for the endogenous vs. dietary forms of body fat accrual. Direct measurement of the de novo lipogenesis fractional contribution to adipose triglycerides does not reveal the true proportional contribution because the total triglyceride deposition rate must be known. For example, if the measured value reveals that 5% of adipose palmitate derived from the de novo pathway, this might represent 100% of newly synthesized palmitate (if only 5% of the total TG in the tissue was new) or 5% of newly synthesized palmitate (if 100% of the total TG in the tissue were new). Correcting the de novo lipogenic contribution for the replacement fraction of

containers. Subjects then took 50 mL of 70%  $^2\text{H}_2\text{O}$  three times a day for 5 days, then 35-50 mL twice-a-day for the remainder of the 8-10 week labeling protocol. This protocol achieves near-plateau body  $^2\text{H}_2\text{O}$  enrichments (1.5-2.0%, see below) within 5-7 days in most subjects and was well-tolerated. Subjects received the  $^2\text{H}_2\text{O}$  as individual aliquots (35-50mL of 70%  $^2\text{H}_2\text{O}$ ) in plastic vials, which were stored in the refrigerator.

[00224] Compliance with outpatient  $^2\text{H}_2\text{O}$  intake was checked through weekly visits (for urine and saliva collection) and by return of vials for counting.

*Collection of body water samples and blood for monocytes*

[00225] Plasma or urine samples were collected weekly in all subjects and frozen in closed containers. Blood was collected in Ficoll-Hypaque solution and the mononuclear fraction removed, after centrifugation. Blood monocytes were isolated as  $\text{CD14}^+$  cells by immunomagnetic beads.

*Adipose tissue sampling protocol and isolation of mature adipocyte-enriched fraction*

[00226] Adipose tissue aspiration biopsies were performed at weeks 5 and 9 of  $^2\text{H}_2\text{O}$  intake, using the procedure described elsewhere (Neese, R., L. Misell, S. Turner, A. Chu, J. Kim, D. Cesar, R. Hoh, F. Antelo, A. Strawford, J.M. McCune, and M. Hellerstein. Measurement in vivo of proliferation rates of slow turnover cells by  $^2\text{H}_2\text{O}$  labeling of the deoxyribose moiety of DNA. *Proc Natl Acad Sci USA* 99(24):15345-50, 2002). In brief, three subcutaneous sites were sampled at each visit – the gluteal region (buttocks), femoral region (thigh), and flank region (mid-back). For the fat aspiration procedure, lidocaine topical anesthetic (1%, with epinephrine 1:100,000) was used. Subcutaneous fat was aspirated with a 14-gauge needle into a 3 cc syringe and then placed in sterile tubes over ice for processing (on the same day). Tissue samples were minced with a sharp blade then treated with type 2 collagenase (Worthington, Lakewood NJ). One mL of a 2 units/ $\mu\text{L}$  solution was added to each adipose sample and allowed to incubate for 1 hr at 37°C. The cell suspension was then poured slowly over a 350  $\mu\text{m}$  mesh filter (Spectrum Laboratories, Rancho Dominguez CA). Adipose cells in the filtrate were then collected and processed for lipid isolation and microscopic analysis.

described elsewhere (Hellerstein, M. K., M. Christiansen, S. Kaempfer, C. Kletke, K. Wu, J. S. Reid, K. Mulligan, N. S. Hellerstein, and C. H. Shackleton. Measurement of de novo hepatic lipogenesis in humans using stable isotopes. *J Clin Invest* 87: 1841-52, 1991).

[00231] Tetrabromoethane was analyzed using a DB-225 fused silica column, monitoring  $m/z$  265 and 266 ( $M_0$  and  $M_1$  masses of the  $^{79}\text{Br}^{79}\text{Br}^{81}\text{Br}$  [parent-OAc] isotopomer). Standard curves of known enrichment were run before and after each group of samples to calculate isotope enrichment.

#### GC/MS analytic procedures

[00232] For all GC/MS analyses, enriched samples were abundance-matched with baseline (unenriched) samples. The abundance range used was that which gave values within 1-2% of theoretical mass isotopomer ratios, as described elsewhere (Neese, R. A., S. Q. Siler, D. Cesar, F. Antelo, D. Lee, L. Misell, K. Patel, S. Tehrani, P. Shah, and M. K. Hellerstein. Advances in the stable isotope-mass spectrometric measurement of DNA synthesis and cell proliferation. *Anal Biochem* 298: 189-95, 2001.). Only analytic runs for which baseline abundances achieved these accuracy levels and for which samples fell within this abundance range were considered acceptable for use in calculations.

#### Statistical analyses

[00233] Group comparisons were by ANOVA. Statistically significant differences were taken to be  $p < 0.05$ . Sources of variability in the measurements were assessed by using random effects models. These included random person effects (that reflect between-person variability), random depot effects within each person (that reflect depot-to-depot variation) and residual week-to-week effects (that reflect week-to-week variability within each depot for each subject). These models also included a fixed week effect to account for systematic change over time. Correlations between adipose TG kinetic parameters and standard (non-kinetic) parameters were also analyzed, using regression models. Pearson correlation and Spearman rank correlation coefficients were calculated for fractional TG synthesis, absolute TG synthesis, DNL and lipolysis vs plasma insulin, glucose, and triglyceride concentrations, percent body fat, total body fat, and waist:hip ratio.



Contribution from DNL to adipose tissue TG

[00238] Incorporation into TG-FA increased in a roughly linear manner between weeks 0 to 9. Fractional DNL (fDNL) reached an average of  $0.020 \pm 0.012$  in gluteal fat (n=17),  $0.023 \pm 0.016$  in flank fat (n=17) and  $0.025 \pm 0.013$  in thigh fat (n=12), after 5 weeks of  $^2\text{H}_2\text{O}$  labeling. After 9 weeks of  $^2\text{H}_2\text{O}$  labeling, fDNL values were  $0.040 \pm 0.025$  in gluteal fat (n=8),  $0.041 \pm 0.024$  in flank fat (n=6) and  $0.041 \pm 0.024$  in thigh fat (n=8). When corrected for the fraction of adipose TG that was newly deposited (i.e., correcting for TG-glycerol synthesis), the fractional contribution from DNL to newly deposited TG-palmitate was relatively constant over time and among depots within most individuals with an average value of about 20%. Individuals appeared to be on their own characteristic curve, however, with consistent results within each individual over time and for all depots tested.

[00239] The approach described in this Example allows concurrent (i.e., simultaneous) measurement of adipose tissue TG synthesis, net lipolysis (TG breakdown), and contribution from the DNL pathway integrated over long periods of time (i.e., weeks or months) in human subjects. Moreover, because outpatient intake of heavy water is simple, easy to comply with and relatively inexpensive, this approach has a number of practical advantages over alternative methods for estimating dynamics of adipose tissue components in humans.

**EXAMPLE 9: Effects of aerobic exercise on mitochondrial cardiolipin:DNA synthesis rates.**

[00240] Mitochondrial biogenesis in muscle occurs in response to aerobic exercise (see above). Mitochondrial biomolecules include not only mtDNA but also membranes rich in phospholipids, in particular a molecule relatively unique to mitochondria – cardiolipin (CL). The integrated biogenesis of cellular organelles such as mitochondria requires coordinated synthesis of mtDNA, mitochondrial cardiolipin and mitochondrial proteins. CL contains 3 glycerol moieties and is therefore ideal for the application of  $^2\text{H}_2\text{O}$  labeling of the glycerol moiety of acylglycerides. The initiation of an exercise regimen in rats results in stimulation of mitochondrial CL as well as mtDNA synthesis and the two parameters correlate well. Accordingly, measurement of mitochondrial CL synthesis may be useful (as a more sensitive

**EXAMPLE 10: T cell DNA vs. plasma immunoglobulin synthesis.**

[00243] The cellular immune system and the humoral immune system represent discrete arms of the body's host defense system. The former is reflected by activation and proliferation of T lymphocytes the latter by synthesis of antibodies (immunoglobulins) by B lymphocytes. It is often important to know which arm of the immune system is activated or suppressed in a disease state or by a drug treatment. Comparison of the proliferation rates of T cells (measured from T-cell DNA synthesis) to the synthesis rates of plasma immunoglobulins represents a measure of cellular:humoral immune activation. The molecular flux rates of T cell DNA and immunoglobulins are measured concurrently (i.e., simultaneously) by administering isotope labeled water and detecting T cell DNA and immunoglobulins by mass spectrometry. Antigen-specific T-cells and/or immunoglobulins are also measured as described above.

**EXAMPLE 11: Mammary epithelial cell and endometrial cell proliferation vs. bone collagen breakdown and brain amyloid-beta production during treatment with selective estrogen receptor modulators (SERMs).**

[00244] SERMs are receiving a great deal of attention as potential therapies to improve women's health. The effects of estrogen differ greatly for different tissues, however, and the risk:benefit balance in an individual woman depends upon these opposing actions. Estrogen tends to increase proliferation of mammary epithelial cells and endometrial cells (thereby increasing risk for breast cancer and uterine cancer) while reducing breakdown of bone collagen (thereby reducing the risk of osteoporosis) and possibly reducing the proliferation of brain amyloid-beta protein (thereby reducing the risk of Alzheimer's Disease). SERMs might be designed that oppose the cell proliferative action but stimulate or leave unchanged the other actions. Those agents would then have great public health and commercial utility. A means of screening for the optimal combination of actions would clearly be useful. All the processes noted (replication of mammary epithelia and endometrial cell DNA, kinetics of bone collagen and brain amyloid-beta) are measured concurrently (i.e., simultaneously) as described herein.

**EXAMPLE 12: Dermal collagen vs. elastin synthesis and breakdown in skin photo-aging**

[00245] Skin wrinkles are increased by exposure to sunlight. This area represents a very large commercial field in cosmetic and drug research. The biochemistry of skin wrinkles (photo-aging) is well characterized, and consists of reduced dermal-layer collagen (due to reduced

simple proteolytic treatment; in the second, tape strips with a specially designed adhesive are applied to the skin surface and the outermost non-living tissue is removed a single layer at a time. Labeled keratin begins to appear quickly in whole epidermis upon administration of deuterated water but it takes about two and a half weeks before any label appears at the surface of normal human skin monitored by tape strips. At least 30 sequential tape applications are required to reach the underlying living portion of the epidermis in normal skin.

[00249] Keratins are very insoluble which makes it easy to isolate the keratin fraction from other proteins in the skin. The same procedure works well on both whole epidermis and tape strips. First, the samples are extracted in a high salt buffer containing Triton X-100. This dissolves essentially all epidermal proteins except keratins. Keratins are then solubilized by boiling in sodium dodecyl sulfate. Although hair is also composed of keratins (with a slightly different structure), hair keratins are not solubilized by this method and do not contaminate the samples. Virtually pure skin keratins are produced by this simple extraction.

[00250] Normal mice given deuterated water do not achieve plateau deuterium enrichments in skin keratin for several weeks (Fig. 7). By contrast, Flaky Skin (FSN) mice, a psoriasis model with hyperproliferative skin, approach full keratin turnover in just four days. A similar contrast of normal vs. FSN mice is seen in epidermal keratinocyte proliferation (Fig. 8). Keratin measurements (Fig. 7) and keratinocyte de novo DNA synthesis (Fig. 8) were measured concurrently (i.e., simultaneously). The parallel changes in keratin and keratinocyte kinetics confirm the hyperproliferative state present within the tissue.

#### **EXAMPLE 14: Comparing actions of chemotherapeutic agents in different tissues**

[00251] Male balb/C nu/nu mice were implanted subcutaneously with non-small cell lung carcinoma cells (SW1573) cells in matrigel. Mice were labeled with  $^2\text{H}_2\text{O}$  and treated with increasing doses of gemcitabine (Gem), administered every other day (as shown in Fig. 9). After 5 days, tumor, colon and bone marrow were removed using techniques well known in the art, and as described, supra. Cell proliferation was measured as described, supra. The data indicate that cell proliferation was inhibited in tumor and bone marrow cells in a dose-response manner, whereas colonocytes exhibited little inhibition. De novo DNA synthesis from all three cell types

using a 35 mm filter. De novo DNA synthesis from all three cell types was measured concurrently (i.e., simultaneously) using the methods of the present invention as described, supra. Fig. 11 depicts the results. As shown, paclitaxel exerted an inhibitory effect, in a dose-dependent manner, on tumor cells and colonocytes, but had little or no effect on bone marrow cells.

**EXAMPLE 15: Comparing angiogenesis and tissue cell proliferation in different tissues**

[00255] The methods of the present invention when applied to angiogenesis are applicable to both animal studies and human clinical trials. The methods provide a faster and more accurate technique for evaluating the activity of potential pro-/anti-angiogenesis drugs and their real efficacy in both early drug discovery and more advanced clinical treatment settings.

[00256] The rate of angiogenesis in a tissue is measured by the endothelial cell proliferation rate. Endothelial cell proliferation was quantified by use of the heavy water ( $^2\text{H}_2\text{O}$ ) labeling technique. Various tissues (as indicated) were digested with collagenase (1mg/mL) into a single cell suspension. Endothelial cells were enriched by Percoll gradient centrifugation, followed by FACS (sorting on isolectin and CD31 positive cells).

[00257] More specifically, the kinetics of angiogenesis was measured in liver and tumor xenographs. Balb/Nu mice were transplanted with human breast tumor cells. After labeling with  $^2\text{H}_2\text{O}$ , individual animals were sacrificed, and both tumor tissue and liver tissue were harvested from the same animal. The proliferation rates of tumor cells and tumor endothelial cells (i.e., de novo DNA synthesis) as well as liver cells and liver endothelial cells were measured concurrently (i.e., simultaneously) using the methods of the present invention as described, supra. Fig. 14 depicts the results. In Fig. 14, the proliferation rate of tumor endothelial cells is shown to be significantly higher than the rate of proliferation of liver endothelial cells. Similarly, the rate of proliferation of tumor cells was greater than the rate for liver cells (Fig. 14). The skilled artisan will realize that this technique can be used to identify angiogenesis inhibitors (i.e., anti-angiogenic compounds). For example, if one or more compounds were directly administered to the tumor xenographs of Balb/Nu mice, then the methods of the present invention would detect an inhibition of tumor endothelial cell proliferation relative to liver endothelial cell proliferation and, potentially a reduction in tumor cell proliferation relative to

boiling for 3 minutes. The dissolved material was size-fractionated by SDS-PAGE. Using standard techniques, proteins were subsequently transferred onto PVDF, and a collagen band corresponding to the alpha monomer of collagen was excised from the resulting membrane after staining the membrane with Coomassie blue.

[00261] Acetone precipitated total liver protein and PVDF-bound collagen were hydrolyzed by treating with 6 N HCl, 16 hours at 110° C. Hydrolysates were dried and the N, O-penatflurobenzyl derivative was generated by addition of PFBBBr (Pierce) at 100° C for 1 hour. The hydroxyl group of hydroxyproline was further derivatized with methyl imidazole/ acetic anhydride. Hydroxyproline was analyzed on a DB225 GC column, starting temp 100°C increasing 10°C / min to 220°C with selected ion monitoring of m/z 352,353.

[00262] Hydroxyproline is a molecule of interest and is measured as OH-proline, the molecule being essentially unique to collagen. Because of this fact, total liver protein hydrolysate can be derivatized and the <sup>2</sup>H enrichment of hydroxyproline determined by GC/MS as described, supra. Fractional synthesis of collagen in normal and CCl<sub>4</sub>-treated animals was calculated from <sup>2</sup>H incorporation into hydroxyproline from total liver protein using the methods of the present invention as described, supra. Fig. 16 depicts the results. Taking Figs. 15 and 16 together, the methods were able to show that significant cell proliferation occurred at both doses of CCl<sub>4</sub>, whereas significant collagen synthesis occurred only at the highest dose of CCl<sub>4</sub> thereby providing a sensitive technique able to distinguish between two biomarkers of liver toxicity.

[00263] Cell proliferation and collagen synthesis (turnover) were also assessed concurrently (i.e., simultaneously) in mice (C57BL/6 and Balb/c) fed griseofulvin. Griseofulvin is recognized as a hepatotoxin, inducing cell proliferation and porphyria. Mice were administered griseofulvin in their chow (1% w/w) for 5 days. After 5 days of treatment cell proliferation (measured as described, supra) in C57BL/6 mice showed increased proliferation relative to controls (Fig. 17). Specifically, administration of griseofulvin at levels below the no observable effect level (NOEL) showed pronounced effects and dose responsiveness after 5 days (Fig. 17). Collagen turnover was also measured as described, supra, and no increase in collagen turnover was detected at the doses of griseofulvin given to the C57BL/6 mice (data not shown).



30 min. Transfer to 15mL conical tube, centrifuge at 1500xg for 3 min, and remove supernatant. Add 2 mL HibernateA, start timer for 3 min, and triturate 10X over 1 min using a barely fire-polished siliconized Pasteur pipet. Allow to settle for 2 min and transfer the supernatant to another 15 mL conical tube

[00268] Repeat above step twice to obtain 6ml cell suspension

[00269] Centrifuge at 1500xg for 3 min and remove supernatant. Resuspend in 10 mL cell dissociation buffer. Incubate for 5 min. Centrifuge at 1500xg for 3 min and remove supernatant.

[00270] Resuspend in 5ml of 4% paraformaldehyde solution. Incubate at RT for 1 hour. Add 5 mL HibernateA and centrifuge at 1500xg for 3 min and remove supernatant. Wash 1x with 5mL HibernateA and centrifuge at 1500xg for 3 min and remove supernatant.

[00271] Resuspend in 550  $\mu$ L HibernateA. Remove 50  $\mu$ L for negative control. Add 5  $\mu$ L rTTC to 500  $\mu$ L cell suspension (1:100 dilution).

[00272] Incubate at RT for 1 hour. Wash 2x with 5 mL HibernateA, centrifuge at 1500xg for 3 min, and remove supernatant. Resuspend in 200  $\mu$ L HibernateA.

[00273] Add 2.5 $\mu$ L anti-TTC antibody to the stained sample, incubate for 30-45 min at RT. Wash 2x with 5 mL HibernateA by centrifuging at 1500xg for 3 min and remove supernatant. Resuspend in 500  $\mu$ L HibernateA.

[00274] Dilute 5  $\mu$ L goat-anti-mouse-ALEXA 488 to 50  $\mu$ L in HibernateA (1:10 dilution). Add 5  $\mu$ L diluted antibody to stained sample and 0.5  $\mu$ L to the negative control. Incubate at RT for 15-30 min.

[00275] Wash 2x with 5mL HibernateA by centrifuging at 1500xg for 3 min and remove supernatant. Resuspend stained sample in 1mL and negative control in 250  $\mu$ L HibernateA. Add 1:500 PI to sample and sort.

[00276] DNA synthesis is measured as described, supra.



in the solvent. Let stand 3 h at room temperature in a dark area. The caps are taken off the glass tubes. The tubes are centrifuged at 2000 RCF for 10 minutes at room temperature. The supernatant (lipid extracts) is poured into 2-mL screw capped vials and the solid residue is discarded.

[00281] 100 mL of developing solvent (chloroform-methanol-water: 69.15%:26.60%:4.26%) is added into the TLC separation tanks 1 h before adding the TLC plates. A 20 mL pipette is used to spot 20 mL of total cerebroside standard on lanes 1, 10, 19 of Whatman LK6DF silica gel 60 TLC plates. For each sample, a 20 mL pipette is used to spot 100  $\mu$ L of lipid extracts on two neighboring lanes (50 mL/lane). Wait until TLC plates look visually dry. The TLC plates are developed in the developing tanks. Each tank holds two plates, facing each other. Normally it takes 40-45 minutes for the plates to be fully developed. After TLC plates develop, wait 15 minutes for the plates to dry. 20 iodine crystals are put into a tank specially used for iodine vapor. The tank is put on a heatblock set at 80 °C. The dried TLC plates are put in the iodine tank to visualize the spots of lipids containing double bonds. The spots of total cerebroside standard are matched with those of samples. The TLC plate images are scanned by a computer. The silica gel is collected onto a weighing box and transferred to a 12 x 75 mm disposable glass tube. 1 mL of chloroform-methanol 2:1 is added with BHT and vortexed. Let stand until silica settles. The solvent is poured into a 13x100 mm screw cap tube. The solid residue is discarded. 1 mL of 3 N methanolic HCl is added into the tube and the tube is capped tightly. The tubes are put on a heatblock at 80 °C for 2 h. The tubes are then removed from the heating block and allowed to cool to room temperature. 1.5 mL H<sub>2</sub>O and 3 mL hexane are added into the tubes and the tubes are vortexed. 1.8 – 2 mL of the bottom layer (methyl glucose and methyl galactose) are transferred to GC vials. The GC vials are put into a fitted rotor of the Jouan 10.10 speedvac and the rotor is balanced and set at 60 °C. Vacuum until the tubes are dry. 100  $\mu$ L of acetic anhydride-pyridine 2:1 (v/v) is added to the GC vials and the vials are covered and allowed to stand for 1 h at room temperature. The vials are then blown down under N<sub>2</sub> until dry. 100  $\mu$ L ethyl acetate is added and the vials are vortexed. The mixture is transferred to GC inserts and the vials are capped with a crimper. The samples are run on the GC/MS and galactocerebroside enrichments are determined as described, supra.

**I claim:**

1. A method of analyzing the relative molecular flux rates of two or more biological molecules in one or more tissues or individuals, comprising:
  - (a) administering isotope labeled water to one or more tissues or individuals for a period of time sufficient for said label to be incorporated in vivo into two or more biological molecules to form two or more isotope-labeled biological molecules;
  - (b) obtaining one or more biological samples from said one or more tissues or individuals, wherein said one or more biological samples together comprise said two or more isotope-labeled biological molecules;
  - (c) measuring the content, rate of incorporation and/or pattern or rate of change in content and/or pattern of isotope labeling of said two or more biological molecules to determine molecular flux rates of said two or more biological molecules; and
  - (d) comparing the molecular flux rates of said two or more biological molecules to analyze the relative molecular flux rates of said two or more biological molecules.
2. The method of claim 1 wherein said isotope labeled water is  $^2\text{H}_2\text{O}$ .
3. The method of claim 1 wherein said isotope labeled water is administered orally, parenterally, subcutaneously, intravascularly, or intraperitoneally.
4. The method of claim 3 wherein said isotope labeled water is administered orally.
5. The method of claim 1 wherein the individual is a human.
6. The method of claim 1 comprising the additional step of discontinuing said administering step (a) prior to performing steps (b), (c) and (d).

19. The method of claim 1 wherein step (c) comprises detecting said two or more biological molecules by liquid scintillation counting, NMR, or mass spectrometry.
20. The method of claim 19 wherein step (c) comprises detecting said two or more biological molecules by mass spectrometry.
21. The method of claim 1 wherein said two or more biological molecules comprises a first biological molecule and a second biological molecule and said first said biological molecule is DNA and said second said biological molecule is a protein.
22. The method of claim 21 wherein said biological sample is obtained from the group chosen from muscle, liver, adrenal tissue, prostate tissue, colon tissue, endometrial tissue, skin, breast tissue, adipose tissue, colon, lymphoid tissue, and brain.
23. The method of claim 21 wherein said DNA and said protein are measured simultaneously.
24. The method of claim 21 wherein said biological sample comprises tumor cells.
25. The method of claim 21 wherein said biological sample comprises bacteria.
26. The method of claim 21 wherein said protein is a cellular protein and said DNA is mitochondrial DNA.
27. The method of claim 21 wherein said protein is a cellular protein and said DNA is genomic DNA.
28. The method of claim 21 wherein said first said biological molecule is adipose tissue acyl-glyceride and said second biological molecule is either protein or DNA.
29. The method of claim 28 further including a third biological molecule wherein said second biological molecule is a protein and said third biological molecule is DNA.
30. The method of claim 1 wherein said one or more biological samples is obtained from somatic tissue.

a) measuring and comparing the relative molecular flux rates of the two or more biological molecules in a first population of one or more tissues or individuals according to the method of claim 1, wherein said first population comprises tissues or individuals lacking said disease, disorder, or condition;

b) measuring and comparing the relative molecular flux rates of said two or more biological molecules in a second population of one or more tissues or individuals according to claim 1; and

c) determining a difference between the molecular flux rates in said first and said second populations to detect, prognose, or monitor the progression of said disease, disorder, or condition.

43. A method of diagnosing, prognosing, or monitoring the progression of interstitial pulmonary fibrosis, comprising:

a) measuring and comparing the molecular flux rates of the two or more biological molecules in a first population of one or more tissues or individuals according to the method of claim 1, wherein said first population comprises tissues or individuals lacking said interstitial pulmonary fibrosis, and wherein said two or more biological molecules comprise lung collagen and fibroblast DNA;

b) measuring and comparing the molecular flux rates of said two or more biological molecules in a second population of one or more tissues or individuals according to the method of claim 1, wherein said two or more biological molecules comprise lung collagen and fibroblast DNA; and

c) determining a difference between the molecular flux rates in said first and said second populations to diagnose, prognose, or monitor the progression of interstitial pulmonary fibrosis.

44. A method of diagnosing, prognosing, or monitoring the progression of hyperlipidemia, comprising:

a) measuring and comparing the molecular flux rates of the two or more biological molecules in a first population of one or more tissues or individuals according to the method of claim 1, wherein said first or second population comprises tissues or individuals without impaired cellular immune activation and wherein said biological molecules T cell DNA and plasma immunoglobulin;

b) measuring and comparing the molecular flux rates of said two or more biological molecules in a second population of one or more tissues or individuals according to the method of claim 1; and

c) determining a difference between the compared molecular flux rates in said first and said second populations, to diagnose, prognose or monitor the progression of impaired cellular immune activation wherein a decrease in the T cell DNA molecular flux rate relative to the plasma immunoglobulin molecular flux rate in said second population compared to said first population identifies the progression of reduced cellular immune activation in said second population.

47. A method of diagnosing, prognosing, or monitoring photoaging (skin wrinkles), comprising:

a) measuring and comparing the molecular flux rates of the two or more biological molecules in a first population of one or more tissues or individuals according to the method of claim 1, wherein said first population comprises tissues or individuals without detectable photoaging and wherein said two or more biological molecules are dermal collagen and dermal elastin;

b) measuring and comparing the molecular flux rates of said two or more biological molecules in a second population of one or more tissues or individuals according to the method of claim 1, wherein said two or more biological molecules are dermal collagen and dermal elastin; and

c) determining a difference between the compared molecular flux rates in said first and said second populations to diagnose, prognose or monitor photoaging, wherein an alteration in the dermal collagen molecular flux rate relative to the dermal elastin molecular

50. A method of determining a tumoricidal or tumoristatic effect of a chemotherapeutic agent, comprising:

a) measuring and comparing the molecular flux rates of the two or more biological molecules in a first population of one or more tissues or individuals according to the method of claim 1, wherein said two or more biological molecules are cellular protein and cellular DNA, and wherein said one or more tissues or individuals of said first population are in need of said chemotherapeutic agent;

b) administering said chemotherapeutic agent to a second population of one or more tissues or individuals; wherein said one or more tissues or individuals of said second population are in need of said chemotherapeutic agent; and

c) measuring and comparing the molecular flux rates of said cellular protein and cellular DNA in said second population according to the method of claim 1, wherein the difference in the relative molecular flux rates between the first population and second population reveals a tumoricidal effect in the case of about equal effects on DNA and protein fluxes, or a tumoristatic effect in the case of greater effects on DNA than proteins, of said chemotherapeutic agent in tissues or individuals in need of said chemotherapeutic agent.

51. A method of determining a tumoricidal or tumoristatic effect of a chemotherapeutic agent, comprising:

a) measuring and comparing the molecular flux rates of the two or more biological molecules in a population of said one or more or more tissues or individuals according to the method of claim 1, wherein said two or more biological molecules are cellular protein and cellular DNA, and wherein said one or more tissues or individuals are in need of said chemotherapeutic agent;

b) administering said chemotherapeutic agent to said individuals or population; and

c) measuring and comparing the molecular flux rates of said two or more biological molecules in said population of one or more tissues or individuals after administration of said



c) measuring and comparing the molecular flux rates of said two or more biological molecules in said population of infectious organisms in said one or more tissues or individuals according to the method of claim 1 to determine the cidal or static effect of said antibiotic.

54. A method of identifying a therapeutic effect of an androgen in one or more tissues or individuals with a wasting or frailty disease or disorder, comprising:

a) measuring and comparing the molecular flux rates of the two or more biological molecules in a first population of one or more tissues or individuals according to the method of claim 1, wherein said first biological molecule is muscle protein or muscle DNA and said second said biological molecule is adipose tissue triglyceride, and wherein said one or more tissues or individuals of said first population are diagnosed with said frailty or wasting disorder;

b) administering said androgen to a second population of one or more tissues or individuals, wherein said one or more tissues or individuals of said second population are diagnosed with said frailty or wasting disorder; and

c) measuring and comparing the relative molecular flux rates of said first and second biological molecules in said second population of said one or more tissues or individuals according to the method of claim 1; and

d) determining a difference between the relative molecular flux rates of said first and said second populations to identify a therapeutic effect of said androgen.

55. A method of identifying a therapeutic effect of an androgen in one or more tissues or individuals with a wasting or frailty disease or disorder, comprising:

a) measuring and comparing the molecular flux rates of the two or more biological molecules in a population of said one or more tissues or individuals according to the method of claim 1, wherein said first biological molecule is muscle protein or muscle DNA and said second biological molecule is adipose tissue triglyceride, and wherein said one or more tissues or individuals of said population are diagnosed with said frailty or wasting disorder;

b) administering said androgen to the population; and

claim 1, wherein said first biological molecule is muscle protein or muscle DNA and said second biological molecule is adipose tissue triglyceride;

b) administering said growth factor or anabolic agent to the population; and

c) measuring and comparing the molecular flux rates of said first and second biological molecules in said population after administration of said growth factor or anabolic agent according to the method of claim 1, wherein an increase in the molecular flux rate of said muscle protein or muscle DNA relative to the molecular flux rate of said adipose tissue triglyceride in said population after administration of said growth factor or anabolic agent identifies a beneficial therapeutic effect of said growth factor or anabolic agent in the wasting or frailty disease or disorder.

58. A method of identifying a beneficial therapeutic effect of a selective estrogen receptor modulator (SERM) in breast cancer therapy, comprising:

a) measuring and comparing the molecular flux rates of the two or more biological molecules in a first population of said one or more tissues or individuals according to the method of claim 1, wherein first biological molecule is mammary epithelial cell DNA or endometrial cell DNA and said second biological molecule is protein or DNA from estrogen-insensitive cells in the breast, wherein said estrogen-insensitive cells are chosen from adipose, fibroblasts, stromal cells, endothelial cells or other non-epithelial cells, and wherein said one or more tissues or individuals of said first population are diagnosed with breast cancer;

b) administering said SERM to a second population of said one or more tissues or individuals, wherein said one or more tissues or individuals of said second population are diagnosed with breast cancer; and

c) measuring and comparing the relative molecular flux rates of said two or more biological molecules in said second population of said one or more tissues or individuals according to the method of claim 1, wherein a decrease in the molecular flux rate of said mammary epithelial cell DNA or mammary endometrial cell DNA relative to the molecular flux rate of said protein or DNA from estrogen-insensitive cells in said second population compared

c) measuring and comparing the relative molecular flux rates of said two or more biological molecules in said second population of said one or more tissues or individuals according to the method of claim 1, wherein an increase in the molecular flux rate of bone collagen relative to the molecular flux rate of said mammary epithelial cell DNA or endometrial cell DNA in said second population compared to said first population identifies a beneficial therapeutic effect of said SERM against osteoporosis.

61. A method of identifying a beneficial therapeutic effect of a selective estrogen receptor modulator (SERM) in osteoporosis therapy, comprising:

a) measuring and comparing the molecular flux rates of the two or more biological molecules in a population of said one or more tissues or individuals according to the method of claim 1, wherein said first biological molecule is bone collagen and said second biological molecule is mammary epithelial cell DNA or endometrial cell DNA, and wherein said one or more tissues or individuals of said population are diagnosed with osteoporosis;

b) administering said SERM to said population of one or more tissues or individuals;  
and

c) measuring and comparing the relative molecular flux rates of said two or more biological molecules in said population after administration of said SERM according to the method of claim 1, wherein an increase in the molecular flux rate of bone collagen relative to the molecular flux rate of said mammary epithelial cell DNA or endometrial cell DNA in the population after administration of said SERM identifies a beneficial therapeutic effect of said SERM against osteoporosis.

62. A method of measuring the effectiveness of a therapeutic agent in treating Alzheimer's disease, comprising:

a) measuring and comparing the molecular flux rates of the two or more biological molecules in a first population of one or more tissues or individuals according to the method of claim 1, wherein said first biological molecule is brain amyloid-beta protein and said second biological molecule is brain 25-hydroxycholesterol, and wherein said one or more tissues are

64. A method of determining a response of muscle tissue to aerobic exercise in an individual, comprising:

a) measuring and comparing the molecular flux rates of the two or more biological molecules according to the method of claim 1, wherein the two or more biological molecules are cellular proteins and mitochondrial DNA and wherein the biological sample comprises muscle tissue;

b) subjecting the individual to aerobic exercise;

c) measuring and comparing the molecular flux rates of said two or more biological molecules according to the method of claim 1, to determine the response of said muscle tissue to said aerobic exercise wherein the biological sample comprises muscle tissue, and wherein an increase in the molecular flux rate of said mitochondrial DNA relative to the molecular flux rate of said cellular proteins identifies increased aerobic fitness.

65. A method of identifying the cause of altered expression of a protein in a tissue or individual, comprising:

a) measuring and comparing the molecular flux rate of the two or more biological molecules according to claim 1 at a first timepoint, wherein said two or more biological molecules are a protein and an mRNA molecule encoding said protein;

b) measuring and comparing the molecular flux rate of said two or more biological molecules according to claim 1 at a second timepoint to identify the cause of altered expression of said protein,

wherein an increase in the molecular flux rate of the protein relative to the mRNA identifies a change in translational control, and

wherein an increase in the molecular flux rate of the protein relative to the mRNA identifies a change in transcriptional control.

66. A method of identifying the cause of a change in total mass or protein expression, comprising:

b) administering said biological agent to said population of one or more tissues or individuals; and

c) measuring and comparing the molecular flux rates of said two or more biological molecules in said population after administration of said biological agent according to the method of claim 1, wherein a difference in the compared molecular flux rates in said population before and after administration of said biological agent identifies a therapeutic property of the biological agent.

69. The method of claim 67, wherein the biological sample is a tissue culture.

70. The method of claim 67, wherein the individual is an experimental animal.

71. The method of claim 67, wherein the individual is a human.

72. The method of claim 67, wherein at least one of said two or more biological molecules is DNA.

73. The method of claim 67, wherein at least one of said two or more biological molecules is a protein.

74. A method of screening a plurality of biological agents in a high-throughput manner according to the method of claim 67.

75. A method of determining one or more toxic effects of a biological agent, comprising:

a) measuring and comparing the molecular flux rates of the two or more biological molecules in a first population of one or more tissues or individuals according to the method of claim 1;

b) administering said biological agent to a second population of one or more tissues or biological molecules; and

c) measuring and comparing the molecular flux rates of said two or more biological molecules in said second population of one or more tissues or individuals according to the method of claim 1, wherein a difference in the compared molecular flux rates of said first

a) measuring and comparing the molecular flux rates of the two or more biological molecules in a population of one or more tissues or individuals according to the method of claim 1;

b) administering one or more compounds to said population; and

c) measuring and comparing the molecular flux rates of said two or more biological molecules in the population of one or more tissues or individuals after administration of said one or more compounds according to the method of claim 1, wherein a difference of the compared molecular flux rates of said two or more biological molecules in said population before and after administration of said one or more compounds identifies a therapeutic target.

79. A kit for measuring and comparing the relative molecular flux rates of two or more biological molecules in one or more tissues or individuals, comprising:

a) labeled water; and

b) one or more tools for administering the labeled water to a tissue or individual, wherein the kit is used to measure and compare the relative molecular flux rates of said two or more biological molecules in said tissue or individual.

80. The kit of claim 79, further comprising chemical compounds for isolating said one or more biological molecules from urine, bone, or muscle.

81. The kit of claim 79, further comprising a tool for administration of precursor molecules.

82. The kit of claim 79, further comprising an instrument for collecting a sample from the subject.

83. An isotopically-perturbed molecule generated by the method according to claim 1.

84. The isotopically-perturbed molecule of claim 83, wherein said molecule is chosen from protein, lipid, nucleic acid, glycosaminoglycan, proteoglycan, porphyrin, and carbohydrate molecules.



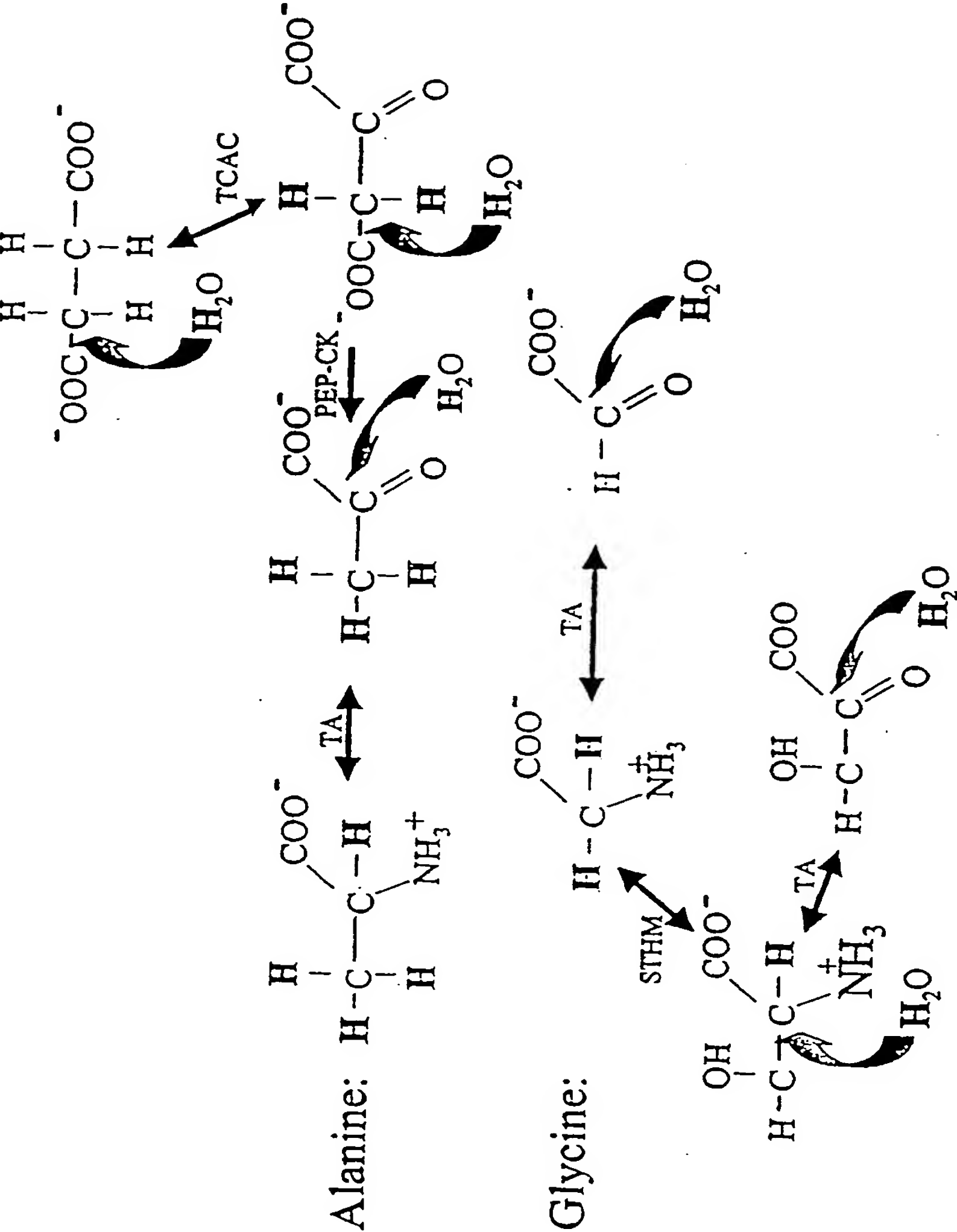


FIG. 1A

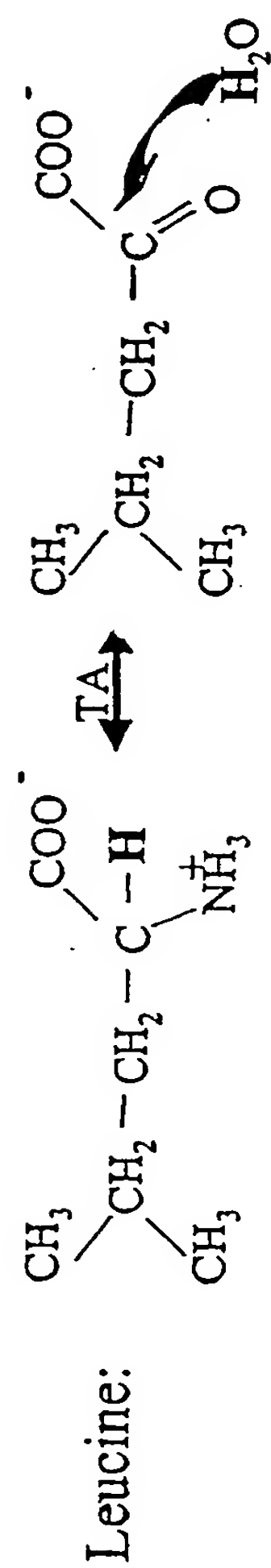


FIG. 1B

# $H_2^{18}O$ Labeling of Free Amino Acid for Protein Synthesis

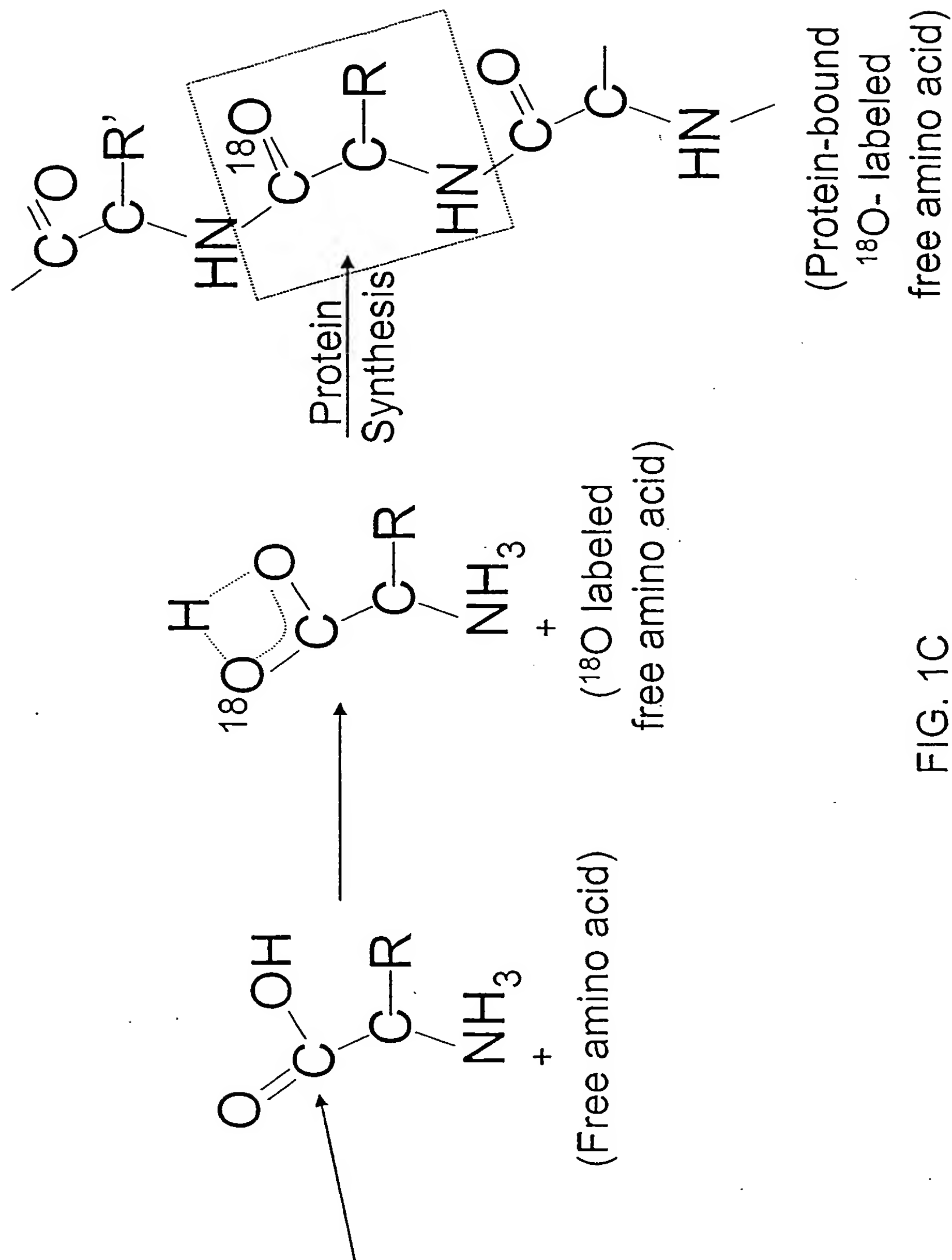


FIG. 1C

# Incorporation of deuterium from water into deoxyribose (dR) of DNA

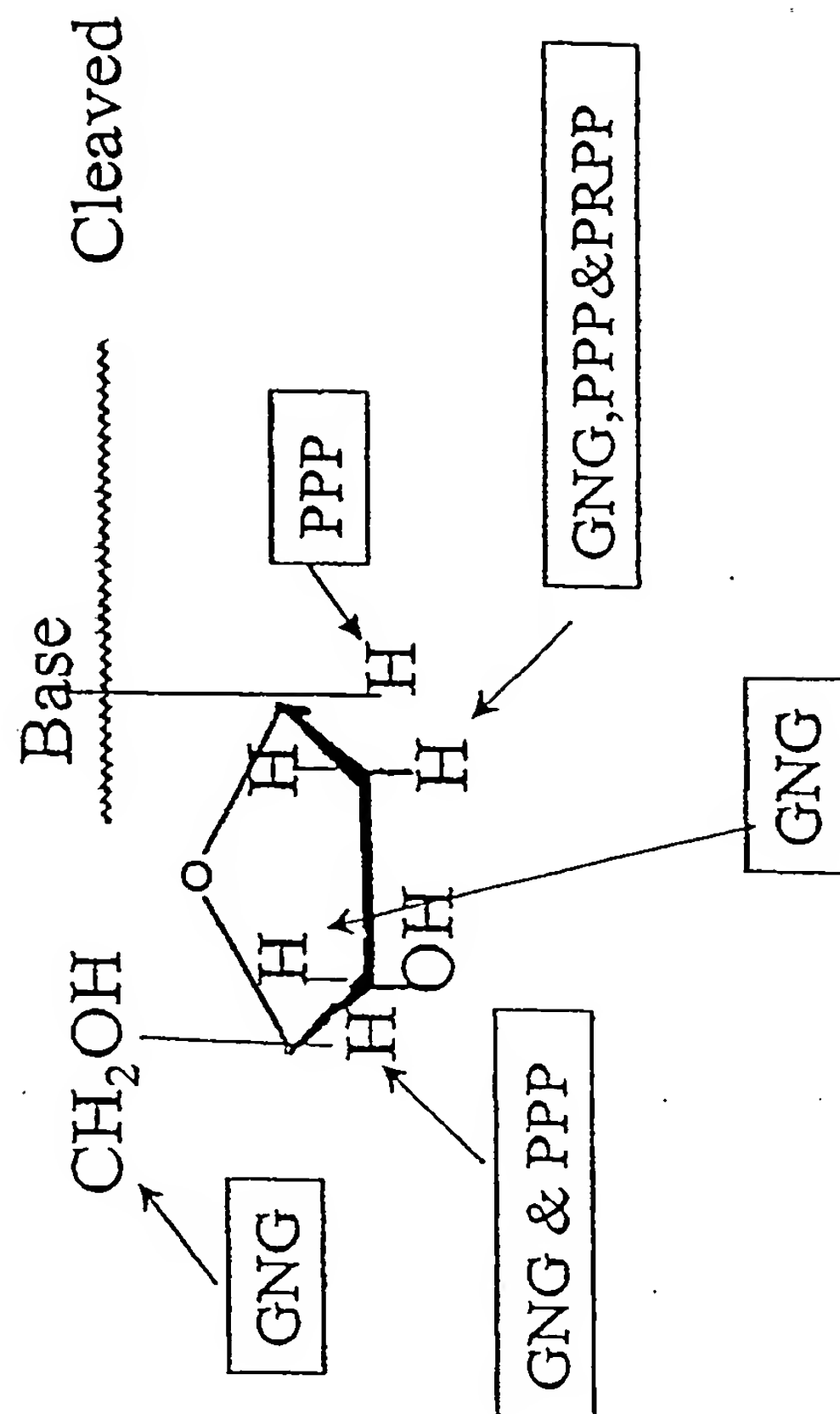


FIGURE 2

5/19

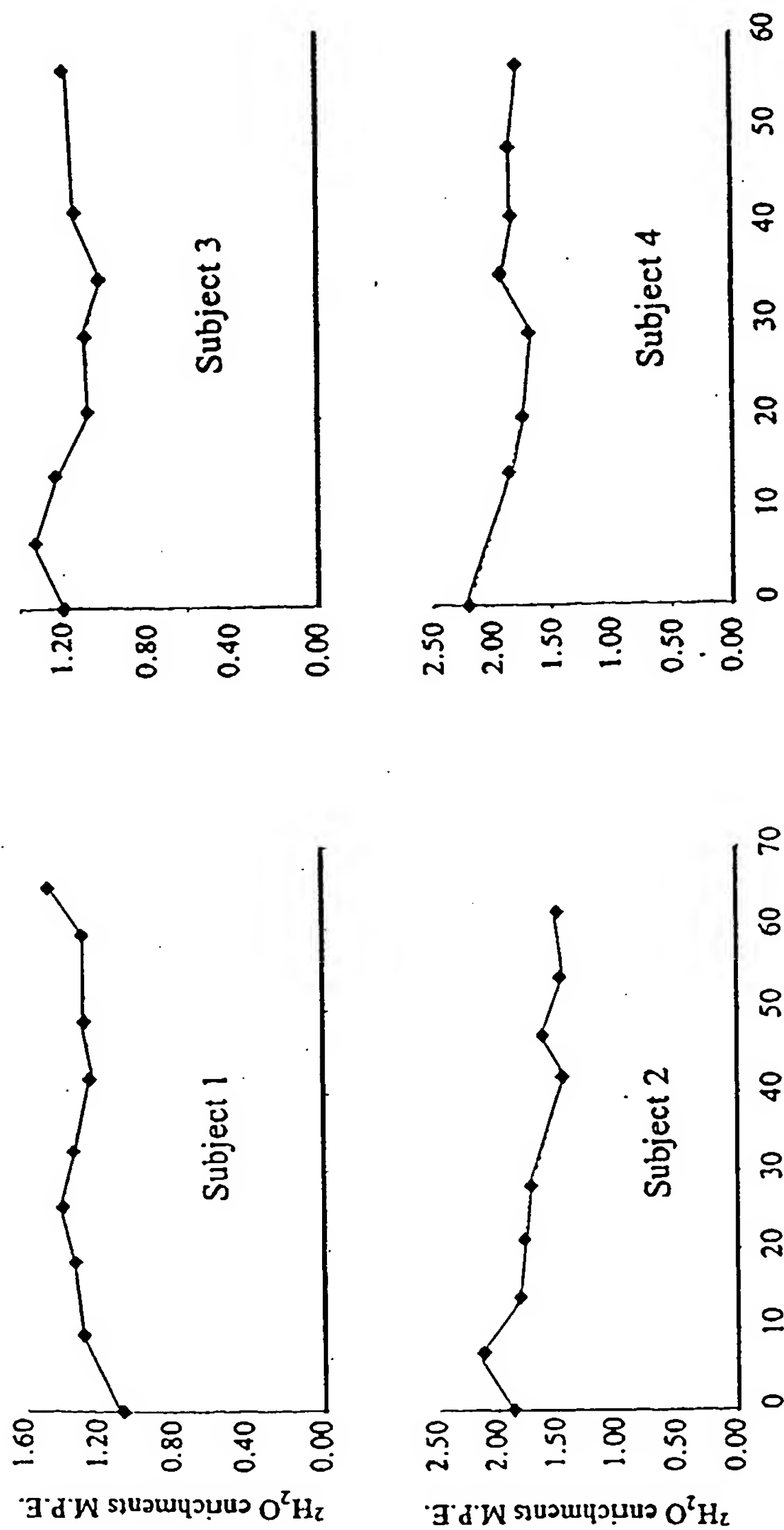


FIG. 3

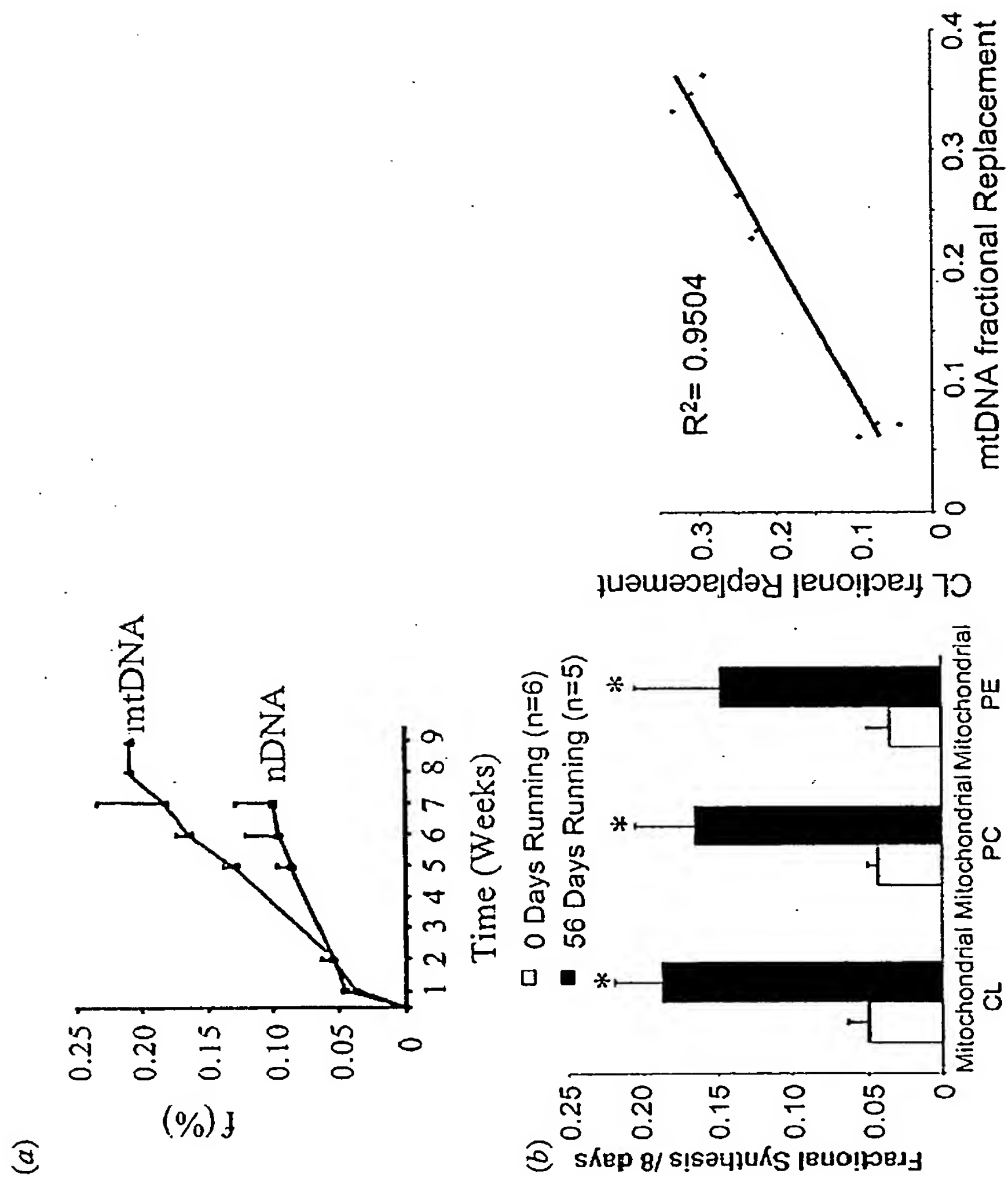


FIG. 4



7/19

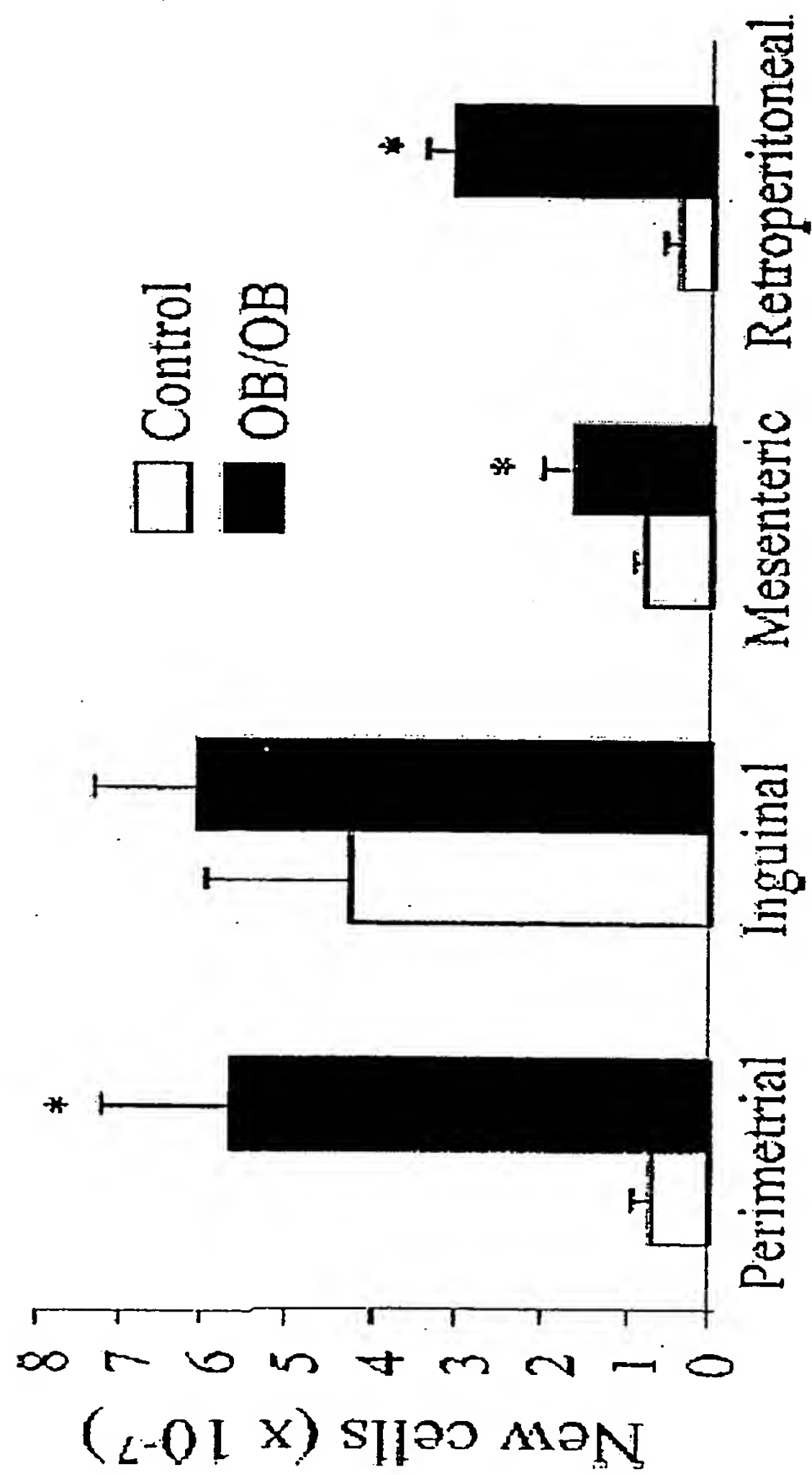


FIG. 5

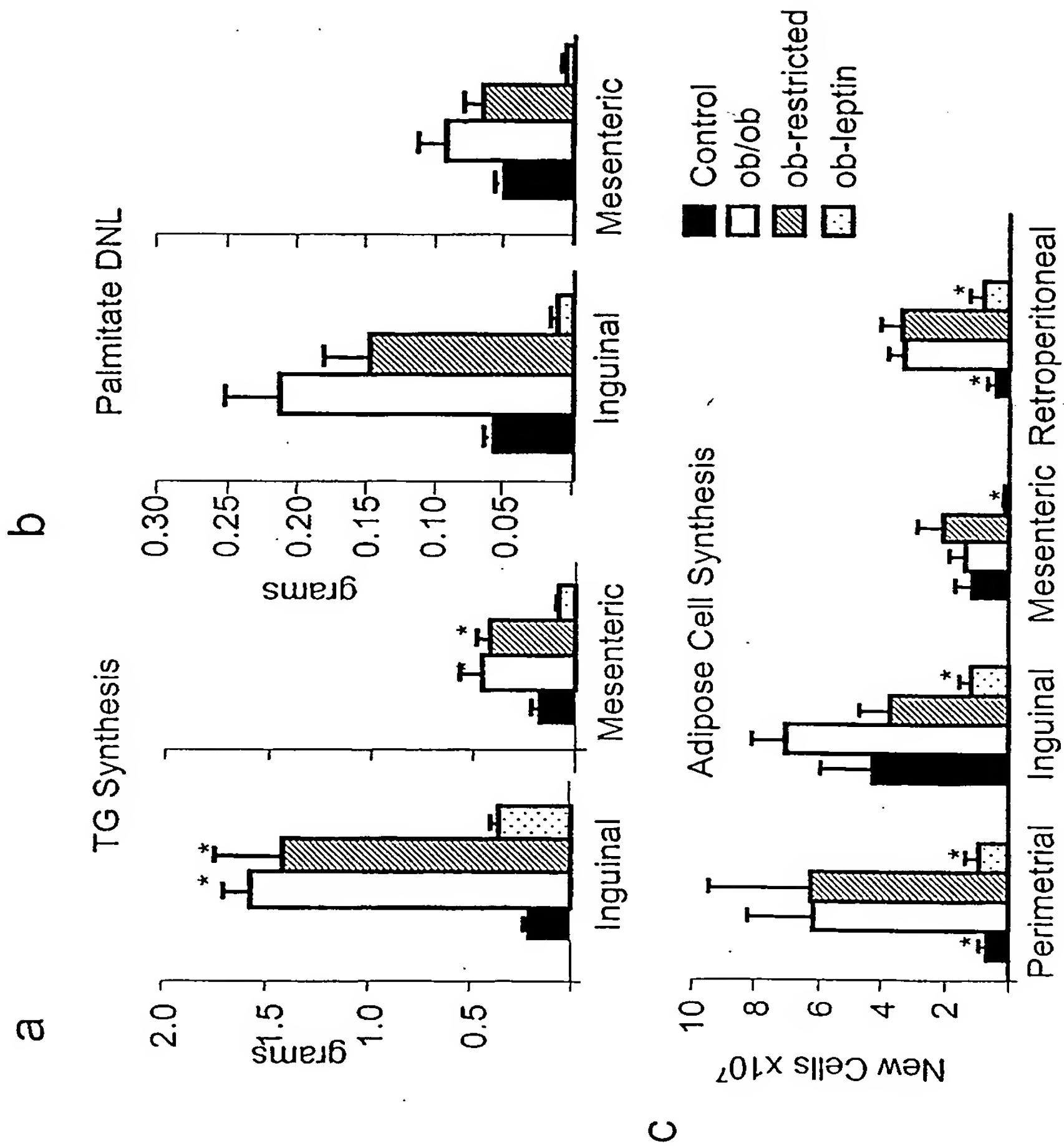


FIG. 6

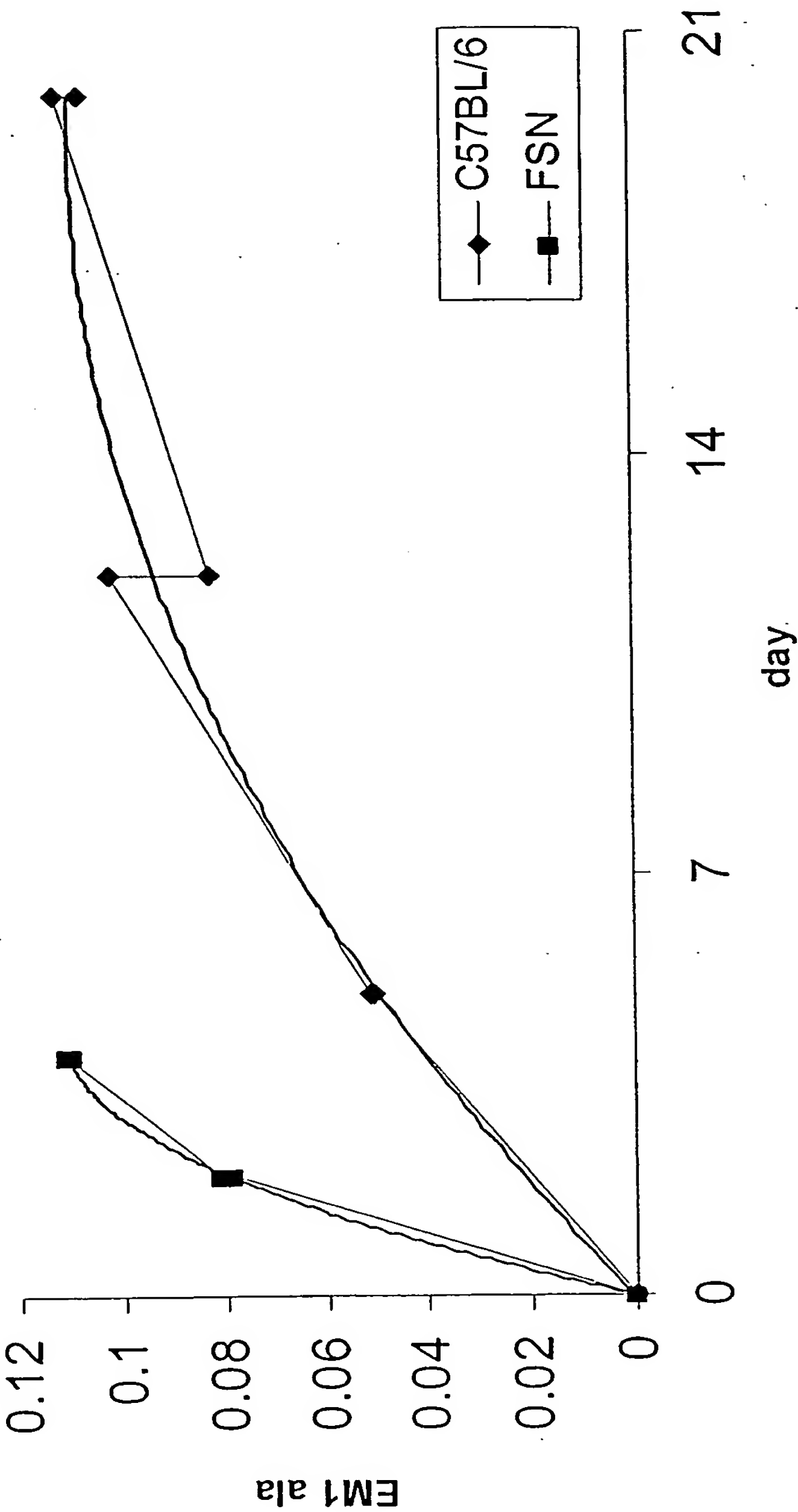


FIG. 7

10/19

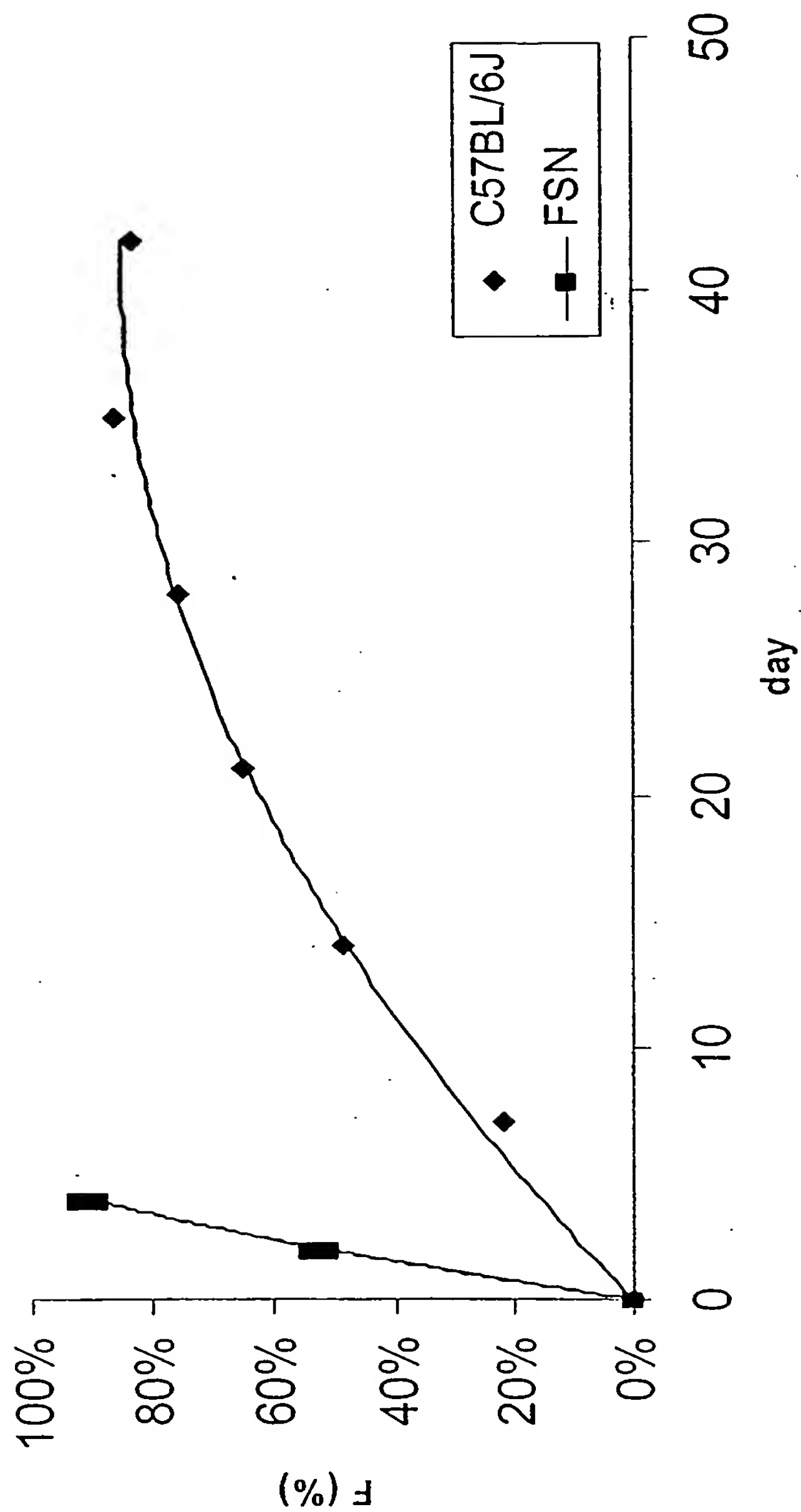


FIG. 8

11/19

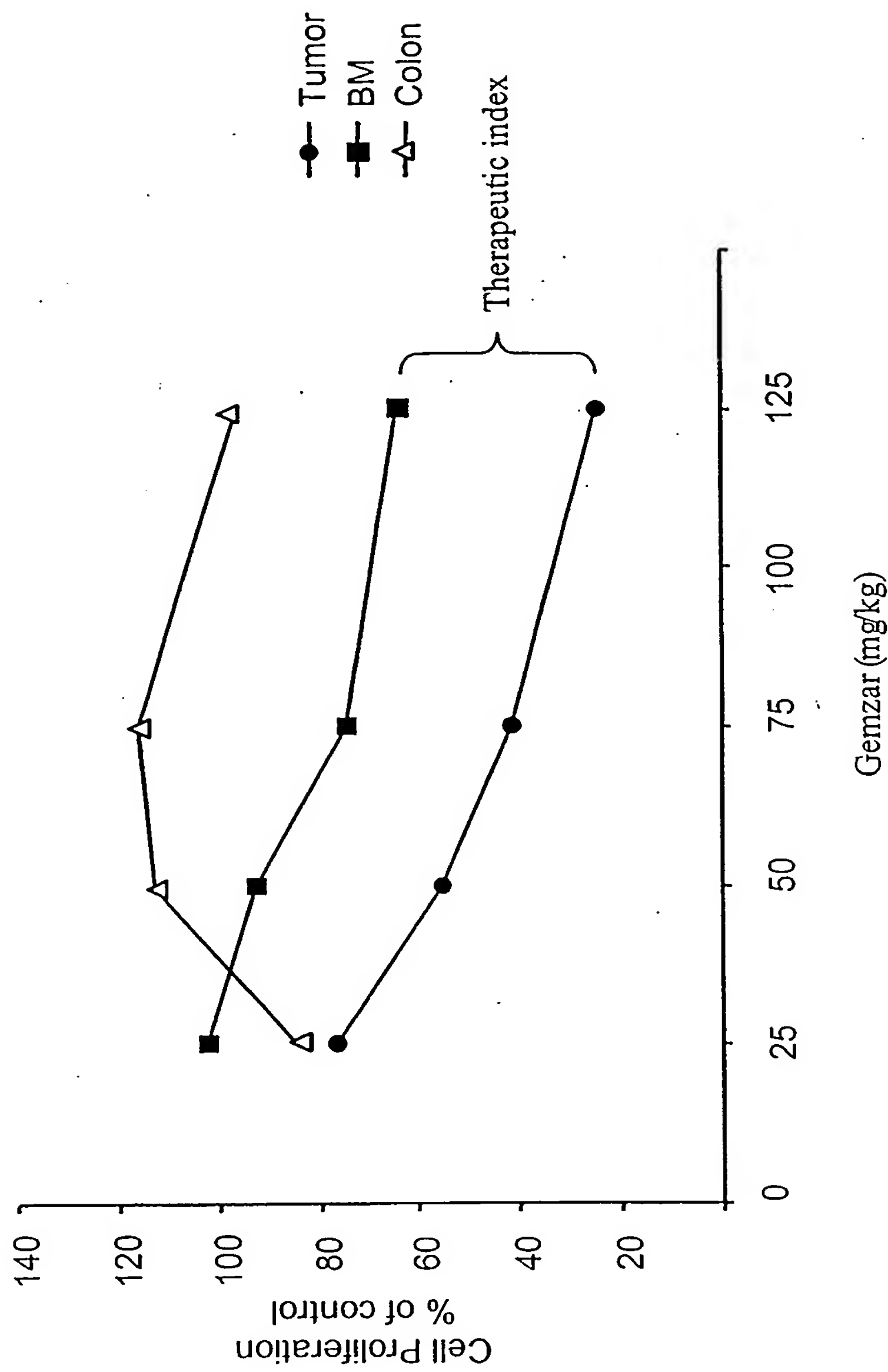


FIG. 9

12/19

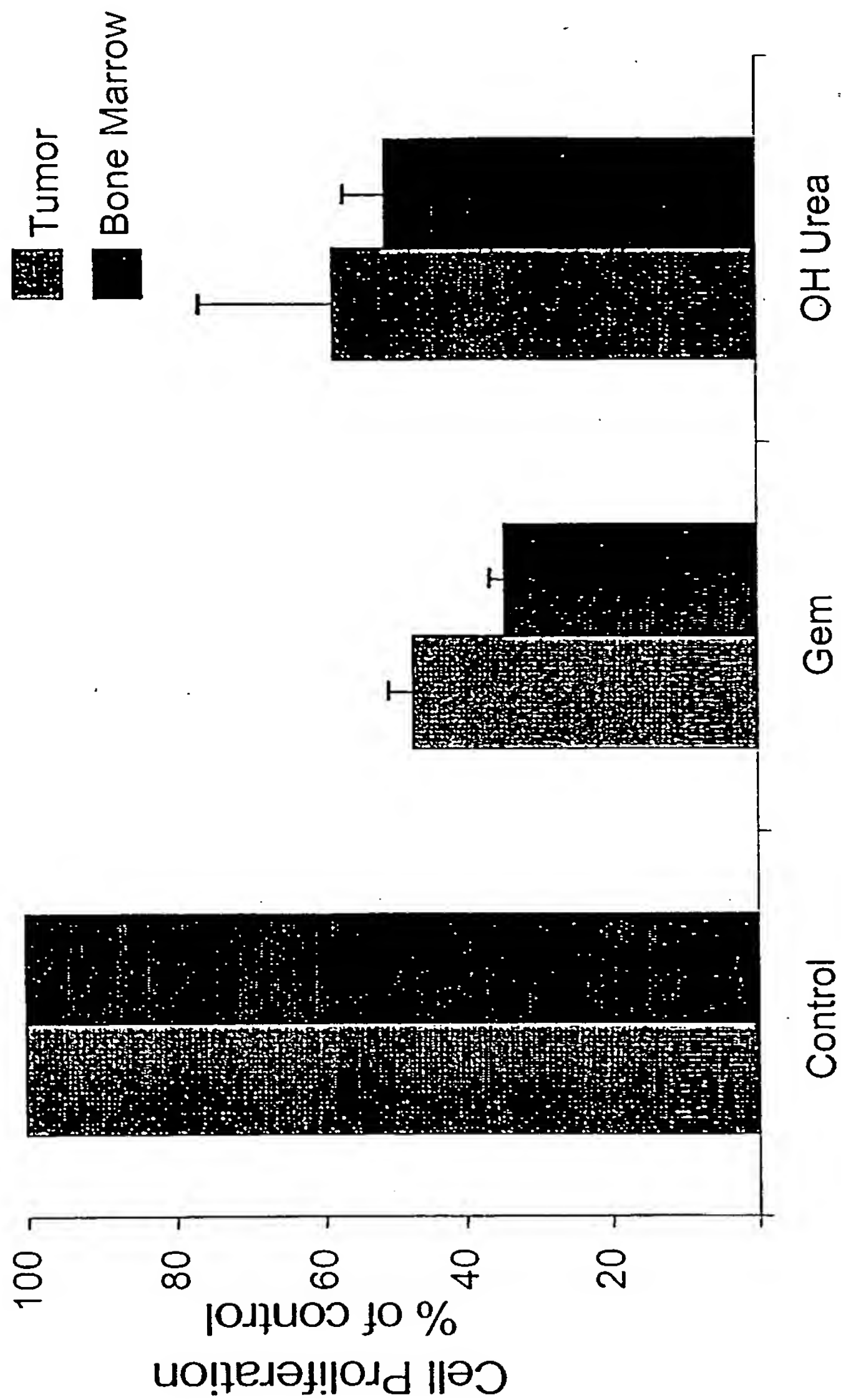


FIG. 10



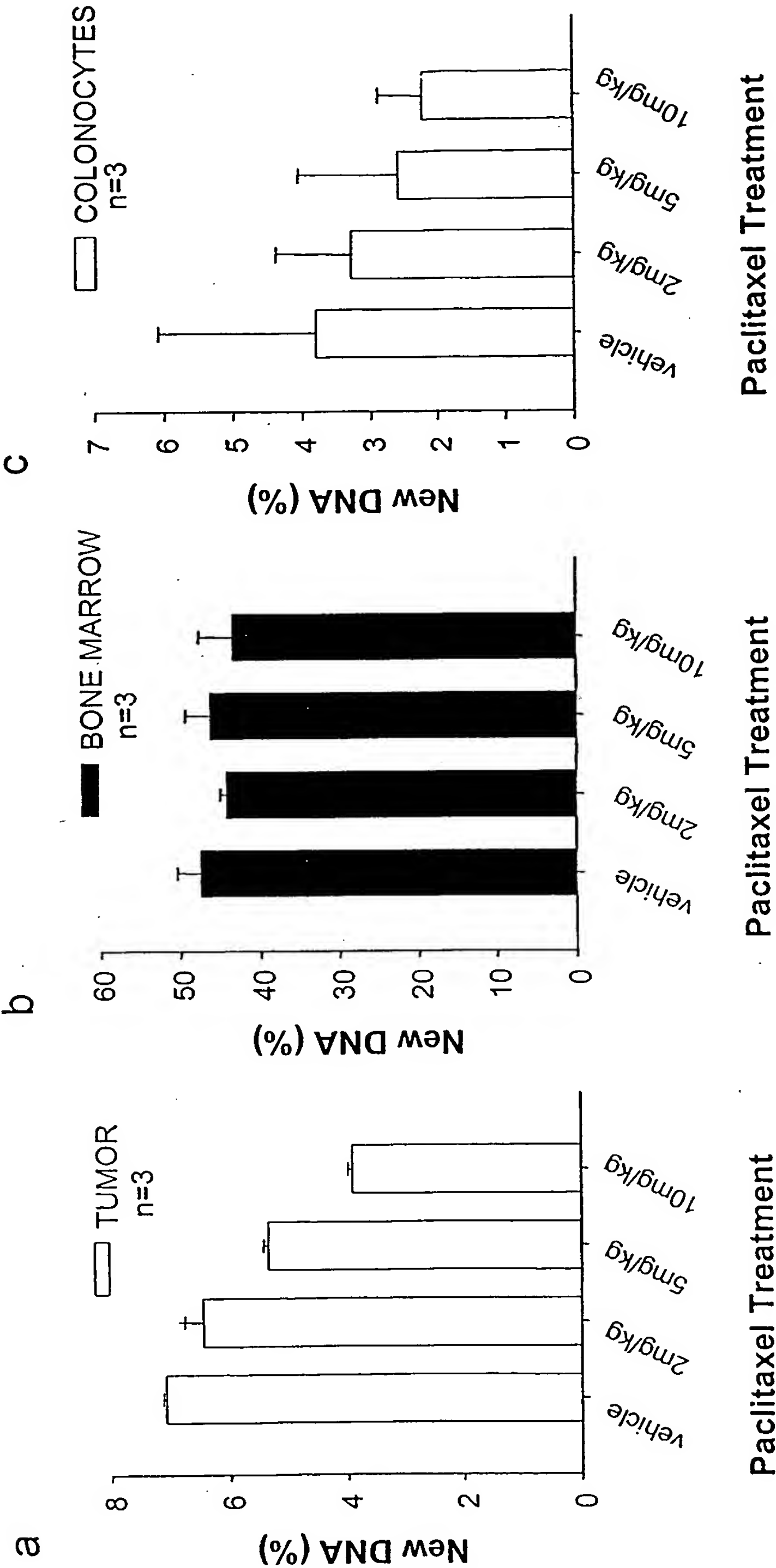


FIG. 11

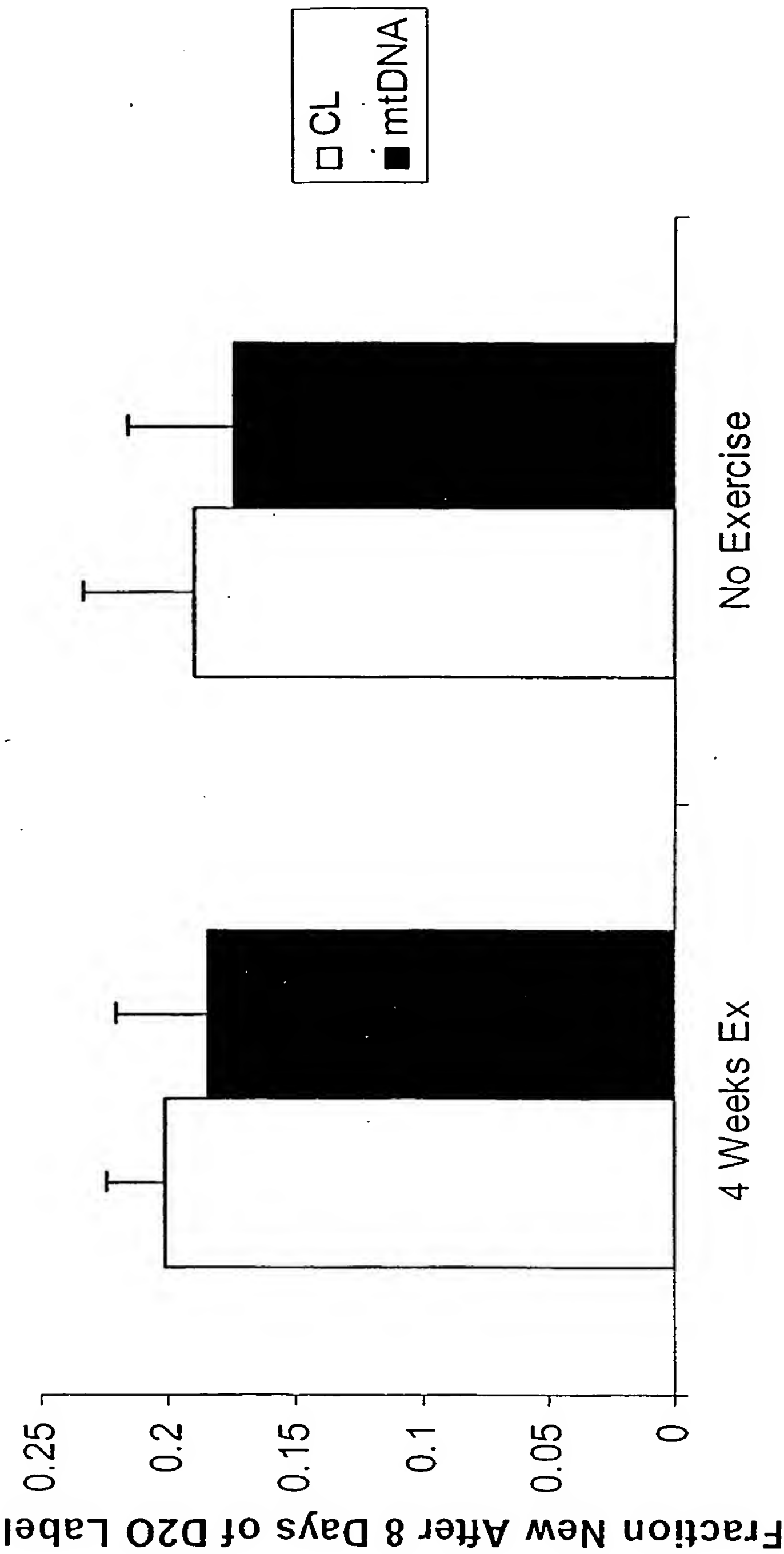


FIG. 12

15/19

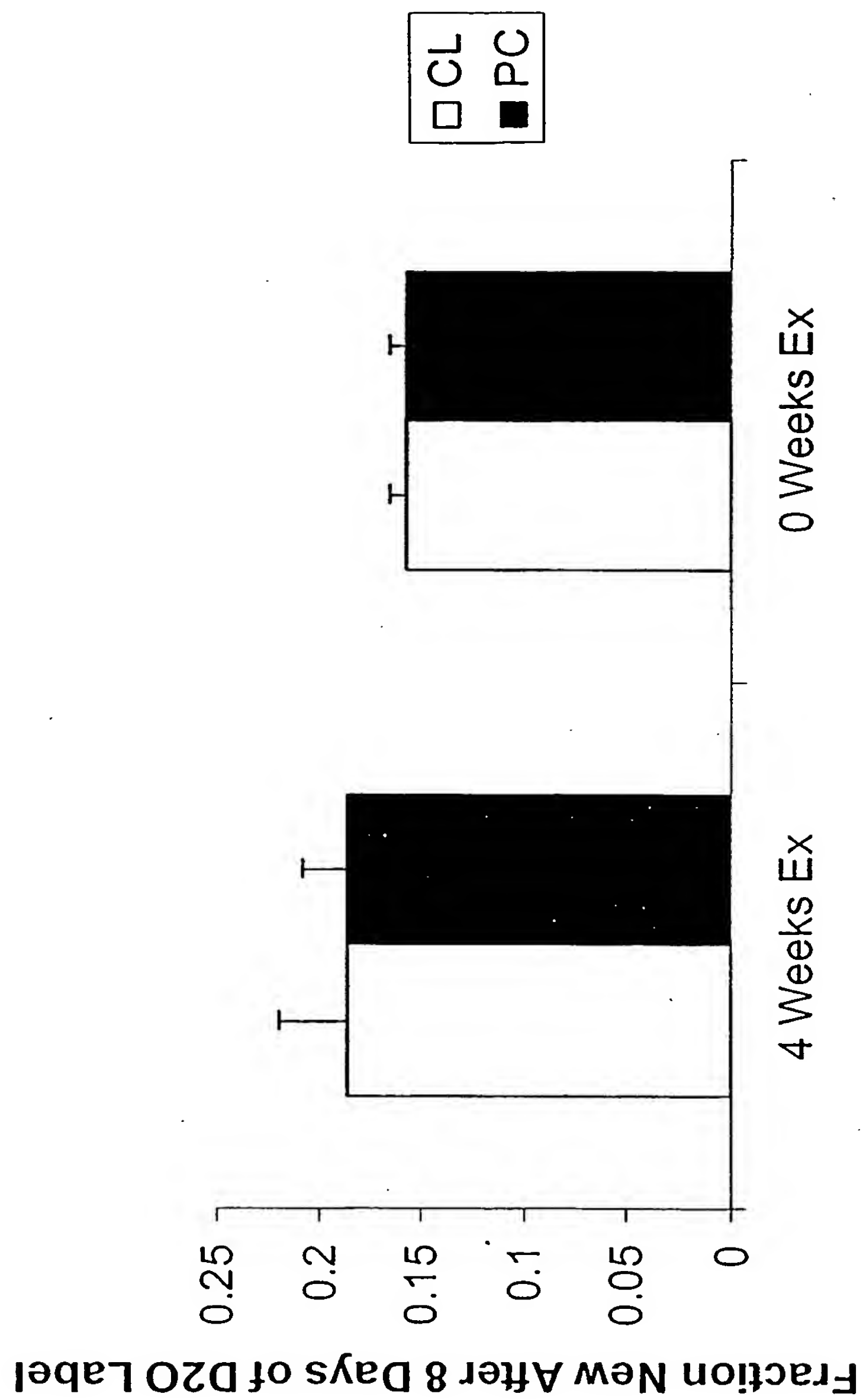


FIG. 13

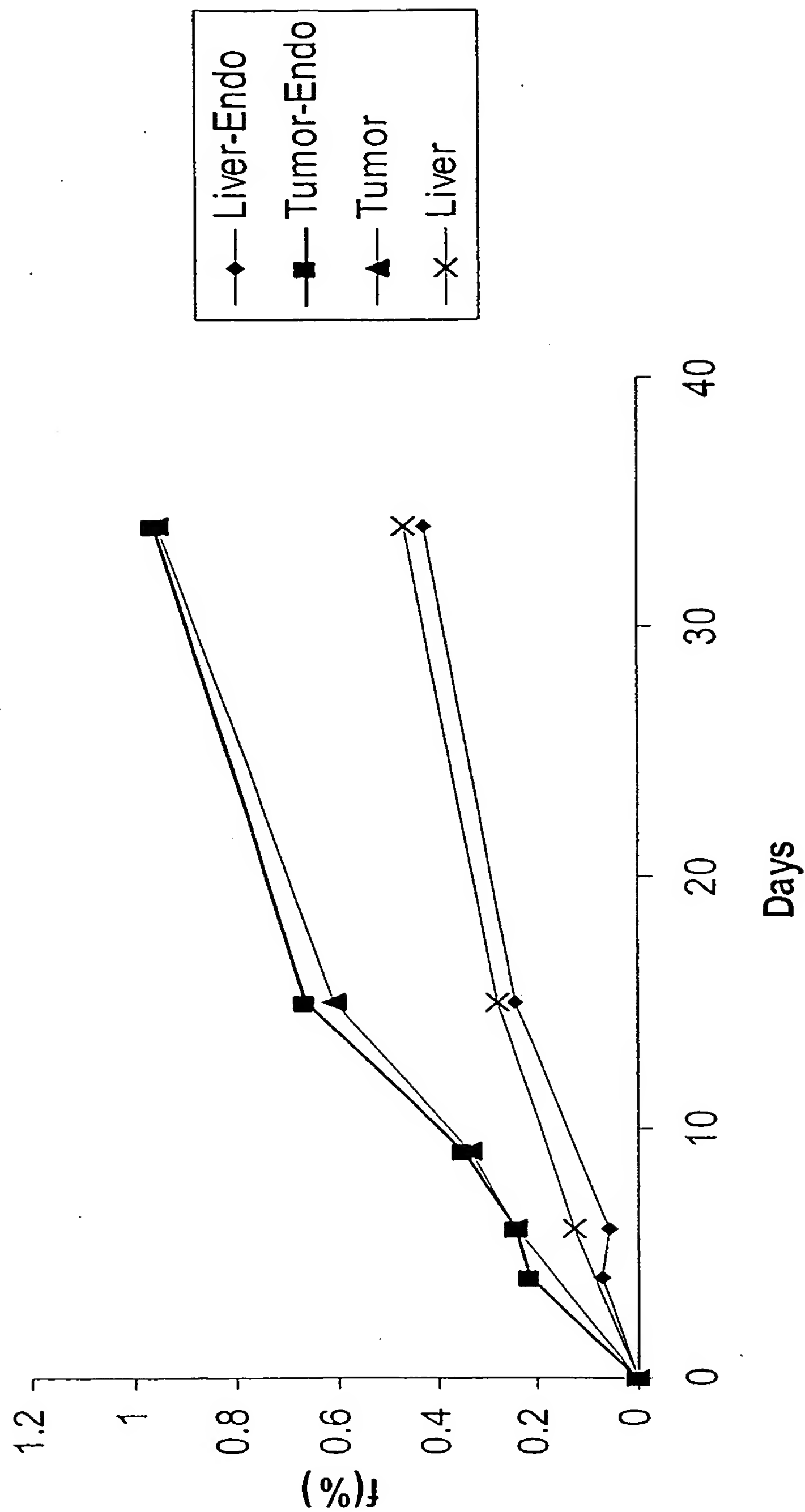


FIG. 14

17/19

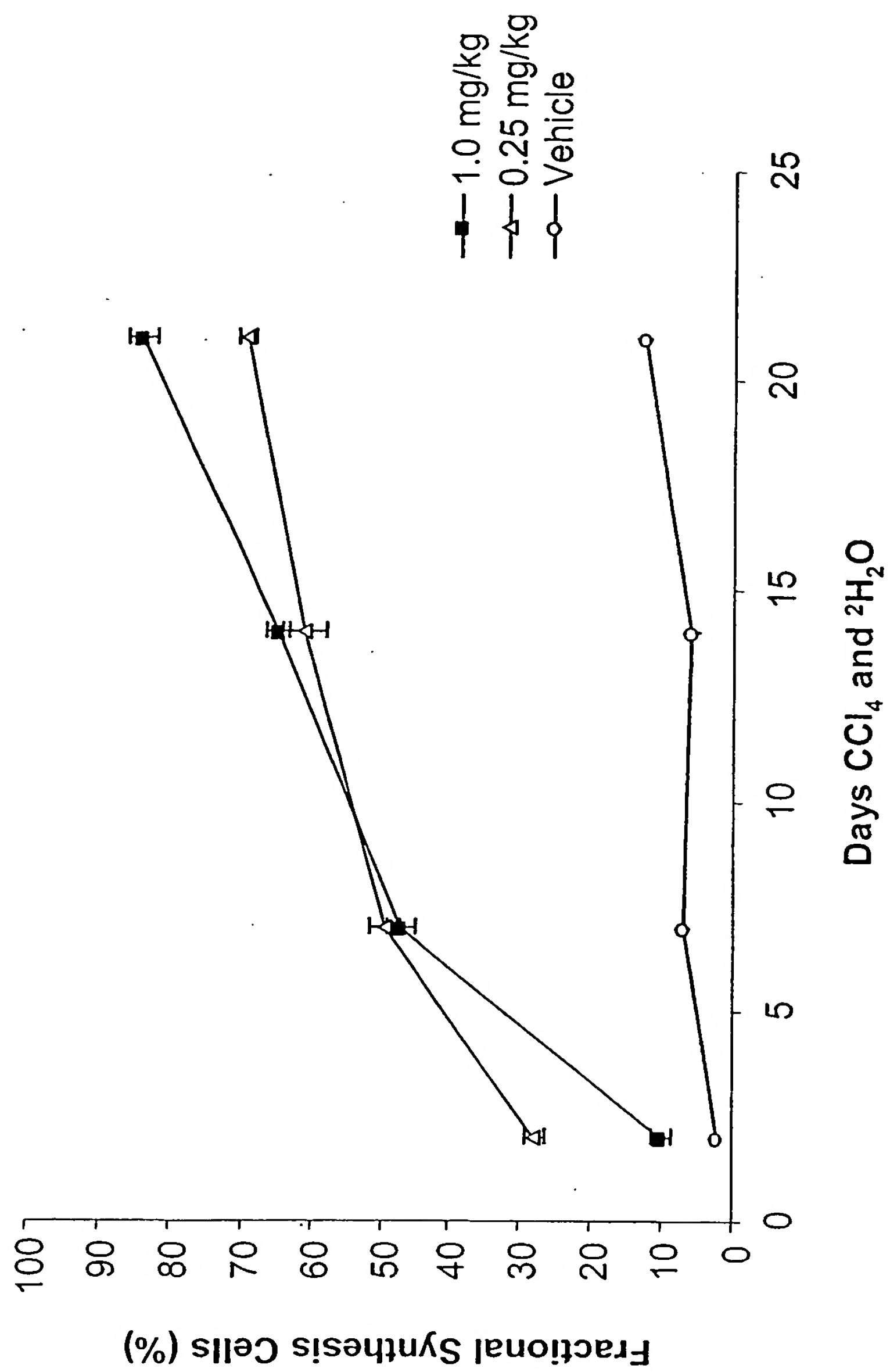


FIG. 15

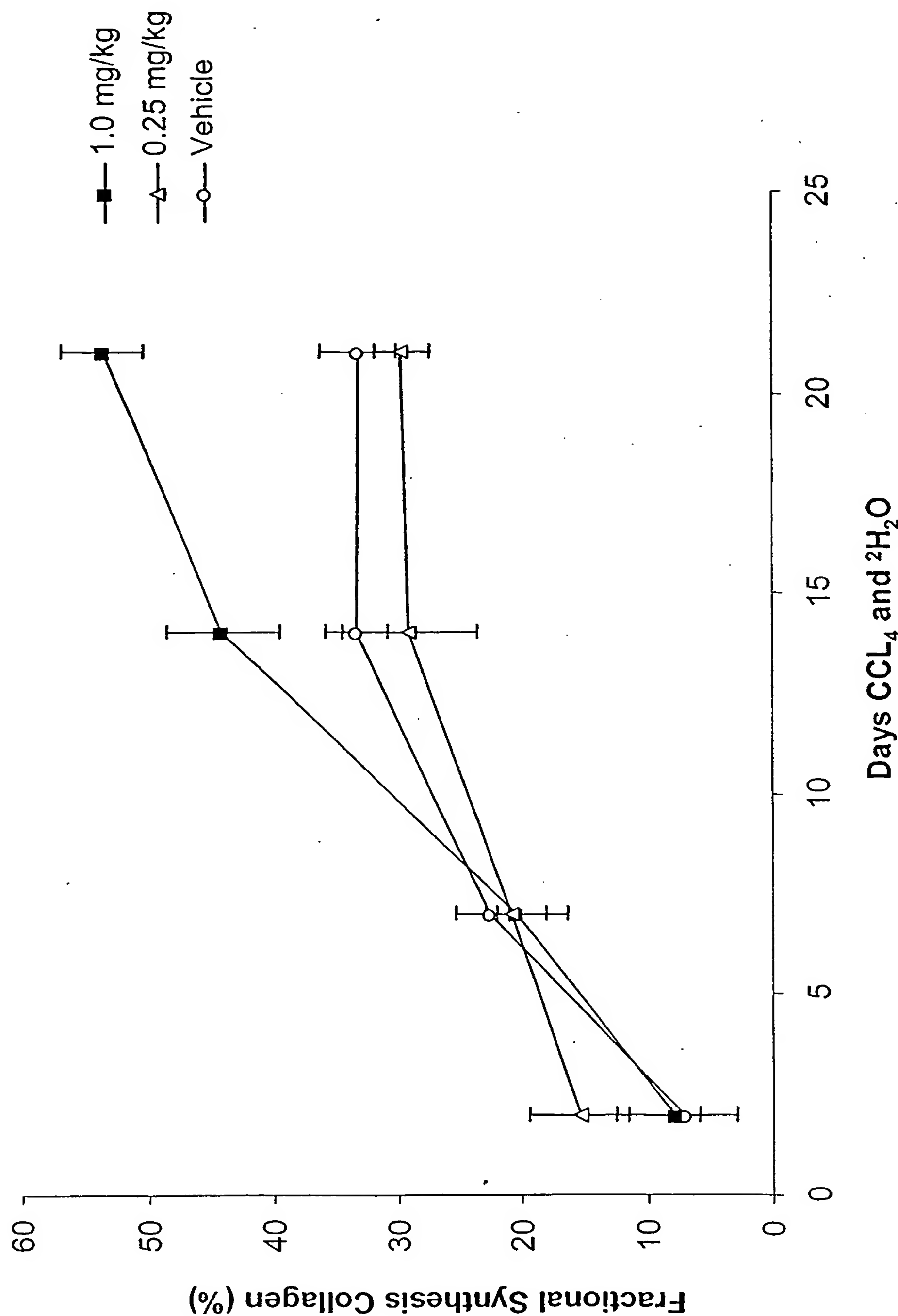


FIG. 16



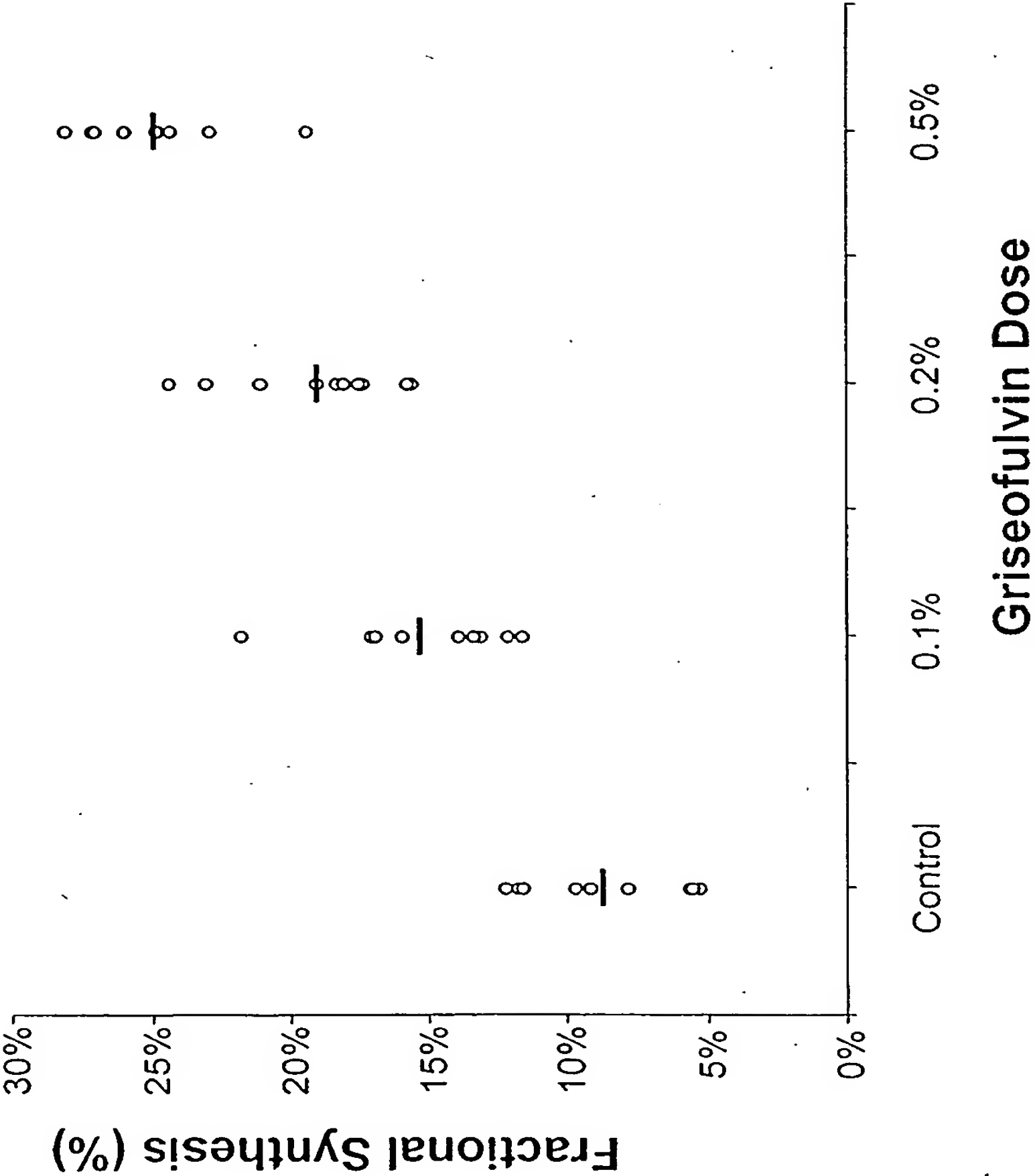


FIG. 17

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